BMJ Open

Study protocol for the randomized controlled trial:
Treatment of early IUGR with low molecular weight heparin
(TRACIP)

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ABSTRACT

Introduction:

Intrauterine growth restriction (IUGR) is defined as a failure to achieve the endorsed growth potential secondary to placental insufficiency. Its incidence is estimated at about 3% of pregnancies and it is associated with 30% of all perinatal mortality and severe morbidity with adverse consequences in neurodevelopmental and cardiovascular health in adult life. Early-onset IUGR represents 20-30% of all IUGR and is highly associated with severe placental insufficiency and with chronic fetal hypoxia, being the major contributor to poor perinatal outcomes.

The available data suggest that low molecular weight heparin has additional effects to its antithrombotic action, improving the placental microvessel structure and function of pregnant women with vascular obstetric complications through the normalization of pro-angiogenic and anti-apoptotic protein levels, cytokines and inflammatory factors at the levels of microvessel of healthy pregnant women, and thus, normalizing the invasion, the angiogenesis activity and the survival of endothelial cells and trophoblast.

The objective of our study is to demonstrate the effectiveness of low molecular weight heparin in prolonging gestation in pregnancies with early-onset fetal growth restriction. Our secondary aims are to demonstrate in these pregnancies the effect of low molecular weight heparin: (i) on reducing neonatal morbidity; (ii) on improving pro-angiogenic and anti-inflammatory maternal and placental; and, (iii) on reducing thrombotic and ischemic placental lesions.

Methods and anlysis: This is a multicenter, triple-blind, parallel-arm randomized clinical trial. Singleton pregnancies qualifying for early (<32 weeks at diagnosis) placental fetal growth restriction (according to Delphi criteria): umbilical artery Doppler with absent/reversed diastolic flow; or estimated fetal weight <10th percentile plus pulsatile umbilical artery Doppler; or estimated fetal weight <10th percentile plus pulsatile uterine artery Doppler) will be randomized to treatment with Bemiparin 3,500 IU / 0.2 ml / day or placebo sc , from inclusion at the moment of diagnosis to the time of delivery.

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Competing interest statement: The authors report no conflict of interest.

Trial registration number: NCT03324139

Authors´contribution:

EM is the project monitor

NM, AP, DO, PIB and JS are coordinators at their respective sites.

EM is the general coordinator and PI of the project.

MC, CR, MDGR and MDT are consultants.

FF is co-principal investigator of the study

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- Preterm IUGR affects a 0.5% of the pregnancies and no treatment has been demonstrated effective.
- This is a multicenter, triple blinded randomized trial with two groups in parallel.
- The primary outcome (prolongation of pregnancy) is a surrogate of perinatal health.
- The limited sample size limits the analysis of secondary outcomes.
- Follow-up of the offspring is limited to the neonatal period.

INTRODUCTION

BACKGROUND

Placental insufficiency and intrauterine growth restriction (IUGR)

Intrauterine growth restriction (IUGR) is defined as a failure to achieve the endorsed growth potential secondary to placental insufficiency. Its incidence is estimated at about 3% of pregnancies and it is associated with 30% of all perinatal mortality and severe morbidity[1] with adverse consequences in neurodevelopmental and cardiovascular health in adult life.

IUGR presents under two different phenotypes: when the onset is early or late in gestation[1]. In general, there is a correspondence between early-onset and the most severe forms of IUGR. Early-onset IUGR represents 20-30% of all IUGR[1] and presents in association with early-preeclampsia (PE) in up to 50% of cases[1]. This type of IUGR is highly associated with severe placental insufficiency and with chronic fetal hypoxia, being the major contributor to poor perinatal outcomes[2].

Placental perfusion depends on the remodeling of maternal spiral arteries by the fetal trophoblast. Maternal spiral arteries supply blood to the placenta and the fetus. To increase up to 10 times the blood supply during pregnancy these arteries become low resistance vessels, a process that depends on a correct interaction between fetal trophoblast and maternal tissues[3].

Fetal cytotrophoblast modifies its epithelial phenotype by one of endothelial cells that allows invading maternal tissues and replacing the endothelium and the muscularis layer of maternal spiral arteries.

The key to the successful implantation of the placenta is the controlled activation of the hemostatic system, which balances the process of trophoblastic invasion and protects against bleeding. In PE and early onset IUGR, this vascular remodeling does not exist or is incomplete[7], which entails a placental hypoperfusion associated with dysfunction of the endothelial cells and the activation of coagulation, fibrin deposits and uteroplacental thrombosis with local implications in the cases of IUGR and systemic implications in the cases of PE[4]. Accordingly, the pathological examination of the placenta of these cases shows uteroplacental thrombosis, infarctions and fibrin deposits.

The natural history of placental insufficiency is towards the loss of fetal well-being, with a progression to hypoxia and acidosis; in clinical practice this deterioration is monitored by the Doppler study of the umbilical artery (which reflects the placental lesion) and the

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ductus venosus (which reflects the fetal cardiac diastolic failure secondary to acidosis) to program the delivery in an elective and adapted way in each case. The latency time to severe fetal deterioration is variable, but on average 3-4 weeks[5]. It is estimated that between 24 and 28 weeks, each week of prolonged gestation increases survival without sequelae by 10-15%; and between 28 and 32 weeks increases by 5%[6]. The objective of fetal monitoring is therefore to maximize gestational age at birth while minimizing the risk of death and sequelae.

Heparin and placenta: biological plausibility

There is in vitro evidence that heparin improves placental perfusion and stimulates neoangiogenesis. On the one hand, heparin exerts its anticoagulant function through the union and potentiation of the antithrombin and, on the other hand, through the mobilization of the tissue inhibitory factor to the circulation[7]. In addition, it has beneficial effects through other routes that do not have to do with its anticoagulant effect[8]: it influences the growth and development of the trophoblast, it diminishes the apoptosis, it acts like an indirect growth factor and it diminishes the inflammation by its anti-complement effects.

In a study[9] comparing the placental microvessels of pregnant women with vascular obstetric complications who had been treated with low molecular weight heparin with healthy pregnant women, it was demonstrated that the effects on invasion, angiogenesis and placental cells survival was similar in these two groups, when normally in the placental microvessels of gestations with vascular complications without heparin treatment, angiogenesis and trophoblastic invasion are decreased and trophoblastic apoptosis increased compared to healthy pregnant women[10].

A significant increase in the levels of some proteins that play an important role in the development and angiogenesis of the placenta has been reported in patients treated with low molecular weight heparin, such as Leptin, Ang-2, VEGFR-3 , Angiostatin, TIMP-1 and TNF-α. Leptin regulates the transfer of nutrients through and into the placenta[11]. Ang-2 stimulates the endothelial cell receptor Tie-2 to regulate vascular remodeling and integrity and it is an important factor in placental biology and vascular development of chorionic villi. VEGFR-3 is localized in endothelial cells and in syncytiotrophoblasts and it has been found significantly diminished in placental levels of IUGR. Angiostatin is an endogenous inhibitor of angiogenesis, which is increased in placentas obtained from women with preeclampsia. TIMP-1 is mainly found in cytotrophoblasts and syncytiotrophoblast of the placenta and decidua. Despite decreased levels of MMP1 in the placenta of women with preeclampsia, no differences were found between these levels of TIMP-1 and placenta levels in healthy pregnant

women[12]. TNF-α participates in systemic inflammation and is released in placental ischemia / hypoxia, as well as macrophages and systemic lymphocytes. Elevated levels of TNF-α have been found in pregnancies with vascular complications [13].

It has also been demonstrated in vitro that heparin recovers MMP-9 levels at the level of placental microvessels[9]. MMP-9 controls trophoblastic invasion and the decrease of its expression in trophoblasts as a response to hypoxia causes reduction in invasiveness, which could impair placenta implantation. Low molecular weight heparin significantly increases the levels of both the pro-MMP form and its active form, which increases the trophoblast invasiveness and promotes angiogenesis in vitro.

Another mechanism of how heparin inhibits placental apoptosis is through the increase of cellular protection mechanism. Levels of Bcl-2, a known inhibitor of apoptosis, have been increased in an in vitro study of heparin treatment of cultured placenta explants, whereas Bcl-2 has decreased levels in syncytiotrophoblast of pregnancies complicated with growth restriction and preeclampsia[14].

Heparin therapy also reduces inflammatory factors (gamma (IFN-γ), TGF-β1, IL-6, IL-8) that have been shown to be elevated in microvessels of pregnant women with vascular complications, normalizing them at the same level as healthy pregnant women.

Much of the available biological evidence comes from in vitro models and the clinical correlation of these effects described in experimental models is poorly characterized. Therefore, the available data suggest that low molecular weight heparin has an additional effect to its antithrombotic action, improving the microvessel structure and function of pregnant women with vascular obstetric complications through the normalization of proangiogenic and antiapoptotic protein levels, cytokines and inflammatory factors at the levels of microvessel of healthy pregnant women, and thus, normalizing the invasion, the angiogenesis activity and the survival of endothelial cells and trophoblast.

Heparin and placenta: clinical evidence

Despite all the biological evidence on its potential benefit, there are few high-quality clinical studies on the efficacy of heparin in gestation.

In a review[15] involving 10 randomized trials with a total of 1139 patients, it appears that in those women at high risk of complications secondary to placental insufficiency, treatment with heparin was associated with a significant reduction in perinatal mortality, prematurity before 34 and 37 weeks and low fetal weight for gestational age. However, these studies only include pregnant women at risk, mainly with history of placental insufficiency in a previous pregnancy (IUGR, preeclampsia, placental abruption or intrauterine death). Another recent meta-analysis[16] (including 6 clinical trials) shows

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that in high-risk pregnant women treated primarily with aspirin, the addition of low molecular weight heparin reduces the incidence of preeclampsia and low birth weight for gestational age by ~ 50%.

Despite the evidence of potential benefit in the secondary prevention of placental insufficiency and its complications, there is little and contradictory evidence about its benefit in pregnant women who already have placental insufficiency. It would be in this clinical indication (which already has a greatly increased risk of complication and need for preterm birth) where the necessary number of patients to treat may prove to be more beneficial. There are 4 clinical trials performed in women with placental insufficiency during gestation, and they present a high heterogeneity of diagnostic criteria and methodological aspects and results. Kingdom et al.[17] and Souza et al.[18] randomized patients with clinical criteria for placental insufficiency who were treated with heparin or placebo and did not achieve any significant difference in gestational age at birth or perinatal morbidity and mortality. In contrast, Yu et al.[19,20] demonstrated significant differences in gestational age at birth and fetal birth weight in patients with placental insufficiency treated with heparin

Justification for the study

There is good biological evidence on the potential beneficial effect of heparin on the placenta, but with insufficient characterization of the clinical correlation of the effects described in experimental models. In addition, the clinical evidence of its benefit to treat gestation already complicated with placental insufficiency is weak and contradictory. This indication of heparin has the potential to prolong gestation in a period in which it is a clinical priority to maximize gestational age at birth to reduce mortality and perinatal sequelae.

HYPOTHESIS

Primary hypothesis

Treatment with low molecular weight heparin at the time of diagnosis of early-onset IUGR prolongs the latency to severe fetal impairment, prolonging the gestational age at delivery of these patients.

Secondary hypothesis

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i. Treatment with low molecular weight heparin at the time of diagnosis of early-onset IUGR reduces neonatal morbidity of these newborns.

ii. Treatment with low molecular weight heparin at the time of diagnosis of IUGR improves maternal and placental biochemical angiogenic and inflammatory profile.

iii. Treatment with low molecular weight heparin at the time of diagnosis of early-onset IUGR reduces placental lesions secondary to poor placental and fetal perfusion.

OBJECTIVES

Primary objectives

To demonstrate the effectiveness of low molecular weight heparin in prolonging gestation in pregnancies with early-onset fetal growth restriction.

Secondary objectives

i. To demonstrate the effectiveness of low molecular weight heparin in reducing neonatal morbidity in pregnancies with early-onset fetal growth restriction.

ii. To demonstrate the effect of low molecular weight heparin in improving pro-angiogenic and anti-inflammatory maternal and placental profile in pregnancies with placental diagnosis of early-onset fetal growth restriction.

iii. To demonstrate the effect of low molecular weight heparin in reducing thrombotic and ischemic placental lesions.

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TRIAL DESIGN

This is a multicenter, triple blinded randomized trial with two groups in parallel in phase IV.

METHODS

STUDY SETTING

The study will be conducted within academic hospitals with specialist experience in managing IUGR in Spain: Hospital Sant Joan de Déu (Barcelona), Hospital Clínic (Barcelona) and Hospital Clínico Lozano Blesa (Zaragoza). The first two hospitals are legally integrated in the Center of Maternal Fetal and Neonatal Medicine of Barcelona (BCNatal). Each of these hospitals is attending over 3000 deliveries and performing over 10000 fetal ultrasounds per year.

ELIGIBILITY CRITERIA
Inclusion criteria:
  I.   Singleton fetus.
  II.   Diagnosis of early-onset IUGR according to Delphi classification[21] (<32 weeks at diagnosis) with umbilical artery Doppler with absent/reversed diastolic flow; or estimated fetal weight <10th percentile plus pulsatile umbilical artery Doppler; or estimated fetal weight <10th percentile plus pulsatile uterine artery Doppler

Exclusion criteria:
  I.   Abnormal karyotype, structural abnormalities or congenital infections.
  II.   Treatment with LMWH, oral anticoagulants or acetylsalicylic acid prior to inclusion.
  III. History of heparin-induced thrombocytopenia.
  IV.   Active hemorrhage or increased risk of bleeding due to changes in hemostasis.
  V.   Severe hepatic or pancreatic dysfunction
  VI.   Organic lesions that can bleed (eg, active peptic ulcer, hemorrhagic stroke, aneurysms or brain tumors).

Withdrawal criteria
  I.   Intolerance or hypersensitivity to bemiparin.
  II.   Allergic reaction.
  III. Clinically relevant bleeding (requiring hospitalization).

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INTERVENTION

Patients who agree to participate and with the informed consent signed will be randomized centrally (website) through the Clinical Trials Unit of Sant Joan de Déu Hospital in Barcelona (block randomization of 10) to 3,500 IU / 0.2 ml / day of Bemiparin sc or placebo of the same presentation as the active drug, from inclusion to delivery (estimated median of 5-6 weeks, with a maximum of 13 weeks).

The treatment will be delivered to the patient weekly, with the same presentation and labeled with the study identification and the participant number.

CONTROL VARIABLES:

1. Maternal age at birth; continuous (years)
2. Smoking during gestation; continuous (cigarettes / day)
3. Maternal weight at the beginning of gestation; continuous (Kg)
4. Maternal height; continuous (cm)
5. Maternal ethnic origin; categorical (Europe, Africa, South America, Maghreb, Asia, Others)
6. Parity (number of deliveries> 22 weeks); discreet
7. History of preeclampsia[22] ; binary (Yes / No)
8. History of gestational hypertension; binary (Yes / No)
9. History of intrauterine growth restricted (neonatal weight below the 10th percentile)[23] ; binary (Yes / No)
10. Gestational age at the inclusion in the study; continuous (weeks).
11. Diastolic blood pressure at inclusion; continuous (mmHg).
12. Systolic blood pressure at inclusion; continuous (mmHg).
13. Mean pulsatility index of the uterine arteries at inclusion and before delivery; continuous (normalized by gestational age)[24].
14. Pulsatility index of umbilical artery at inclusion and before delivery; continuous (normalized by gestational age)[25].
15. Pulsatility index of middle cerebral artery at inclusion and before delivery; continuous (normalized by gestational age)[26].
16. Pulsatility index of ductus venosus at inclusion and before delivery; continuous (normalized by gestational age)[27].

OUTCOMES

Primary outcome

Gestational age at birth (dated by ultrasound <14 weeks by measurement of crown rump length; continuous (mm)[28] .

Secondary outcomes

1. Intrauterine growth restriction: neonatal weight less than the 10th percentile for our population[23] with pulsatility index of the umbilical artery during the third trimester (on two separate occasions> 48h) higher than the 95th percentile[25]; Binary (Yes / No)

2. Preeclampsia: diastolic blood pressure (DBP) >= 90mmHg and / or systolic (SBP)> = 140 in 2 separate determinations> 4h with proteinuria> 300mg / 24h; Binary (Yes / No)

3. Severe preeclampsia: preeclampsia with BP> = 110/160 mmHg, oliguria (<400 ml / 24h), neurological symptoms, acute pulmonary edema, persistent epigastric pain, hepatic dysfunction, analytical signs of hemolysis (LDH> 700 U / L) and / or thrombocytopenia (<100,000 / ml); Binary (Yes / No)

4. Preterm birth before 34 weeks of gestation; binary (Yes / No)

5. Emergent caesarean section for loss of fetal well-being; binary (Yes / No)

6. Neonatal weight; continuous (g)

7. Neonatal acidosis (arterial pH <7.10 + base excess> 12mEq / L); binary (Yes / No)

8. Perinatal mortality (from 22 weeks of gestation to 28 days postpartum); binary (Yes / No)

9.Days in the Neonatal Intensive Care Unit; continuous (days)

10. Neonatal morbidity (seizures, intraventricular hemorrhage grade III or IV, periventricular leukomalacia, hypoxic-ischemic encephalopathy, abnormal electroencephalogram, necrotizing entercholitis, acute renal failure (serum creatinine> 1.5 mg / dL) or heart failure (requiring inotropic agents); binary (Yes/ No)

11. Biomarkers in maternal blood: sFlt and PIGF; continuous (pg/mL)

12. Biomarkers in umbilical cord blood: TNF alpha, IL6, IFN gamma, FGF basic, VEGF and PIGF; continuous

13. mRNAs in trophoblast: IL6, INFgamma, TNF alpha, VEGFA, VEGFB, FGF2 and RQVEGF Receptor1; continuous

PARTICIPANT TIMELINE

Table 1 shows the participant timeline

SAMPLE SIZE

A systematic review (PubMed and ISI Web of Knowledge) of clinical trials published in pregnant women suspected of placental insufficiency involving heparin treatment (both

unfractionated and LMWH) has been performed. The search resulted in only 4 studies whose quality (Newcastle-Ottawa scale) was good or very good. The meta-analysis of these studies demonstrated very high heterogeneity under a fixed-effect model (I^2 90.4%) to estimate the difference in gestational age at birth between groups. Under a random effects model, the mean difference in gestational age at delivery was 1 week with a standard deviation of 0.9. Under these assumptions, the sample needed to detect a clinically relevant difference (fixed at 1 week) at gestational age at delivery with a power of 95% and an alpha risk of 5% was 22 patients per arm (44 patients in total. To compensate for possible withdrawals will require 25 per arm (50 patients).

ALLOCATION

Using an online service (http://www.randomization.com), randomization sequences will be generated in blocks of 10 subjects to assure balanced distribution within study arms, stratified for participating site. The allocation sequence will be sequestered internally by a Clinical Trials Unit (CTU).

At the time of the diagnosis and after enrollment, recruiting physicians will obtain the allocation group from a using a web-based system.

Each treatment pack will only be identified by a randomization code. The treatment allocation will only be revealed to the researchers after completion of the study or where clinically essential.

Pharmacy Department of Sant Joan de Déu Hospital will provide labelling (for all packs and blister sheets) ensuring complete blinding to all investigators and participants in the study, which includes the principal investigator, participating research doctors, project managers and others involved in the trial.

Matching placebo will be identical to the intervention (bemiparin) in such parameters as size, physical properties and appearance. The Pharmacy Department will keep the randomization code list confidential to maintain the concealment.

PARTICIPANT COMPLIANCE

Compliance will be assessed by trial teams by counting remaining injections at each follow-up visit and asking about compliance weekly.

Participants will be encouraged to report any concerns or side effects in a diary for review at each trial visit.

DATA COLLECTION

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Patients will be recruited at the time of diagnosis of early-onset IUGR, at which time the fetal-placental Doppler study is performed routinely (obtaining the mean pulsatility index of the uterine arteries, pulsatility index of the umbilical artery, middle cerebral artery pulsatility index and ductus venosus pulsatility index).

In this first visit also, the control variables defined in the previous section will be collected. Participant data from this first visit and the following visits for this study (anonymized) will be entered into an electronic case report form (CRF) hosted in a secured website by each site coordinator. Logic and range rules operate in the e-CRF to minimize errors in entering the information.

At the time of delivery, maternal blood, cord blood and placenta will be collected. Perinatal outcomes will be collected after delivery.

Obtaining and processing of maternal blood samples:

5mL of maternal blood will be obtained and collected in tubes without anticoagulant. They will be transported to the laboratory in less than 1 hour after extraction, maintaining the safety guidelines for transport of biological material established by each center. Blood tubes will be centrifugated at 1500g for 15 minutes. Supernatant (serum) will be aspirated and will be stored in cryovials, properly labeled and identified in a -80ºC freezer. Levels of PlGF and SFlt-1 (picograms / mL) using Elecsys automated platform by electrochemiluminescence (Cobas analyzers, Roche Diagnostics) will be determined. The intra-assay coefficient of variation is less than 4% and the interassay is 2.4 to 4.6%.

Collection and processing of cord blood samples:

After the fetal extraction, umbilical cord venous blood will be collected with a tube of 10 ml of silicone without additives. They will be transported to the laboratory in less than 1 hour after extraction, maintaining the safety guidelines for transport of biological material established by each center. Blood tubes will be centrifugated at 1500g for 15 minutes. Supernatant (serum) will be aspirated and will be stored in cryovials, properly labeled and identified in a -80ºC freezer until analyzed in duplicate in the LABSCAN 100 Multiplex Analyzer (Luminex) (LUMINEX 100 IS software).

Collection and processing of placental tissue samples:

After delivery, two biopsies of placental tissue will be taken and stored in later RNA in aliquots at -80 ° C until the time of analysis. DNA will be extracted from placental tissue with the chemistry of Flexigene (Qiagen).

Maternal blood, cord blood and placental tissue samples will be all analyzed in the *Centro de Investigación Biomédica de Aragón (CIBA), Aragon Institute for Health Research (IIS Aragón) Zaragoza, Spain*.

DATA MONITORING

An independent Clinical Trial Unit will perform off-line data audit every 6 months checking for missing information and errors.

STATISTICAL ANALYSIS

Analysis will be based on originally assigned groups (intention-to-treat).

The primary objective will be analyzed by:

I) Univariate analysis:

    A. By comparison of gestational age in each group with student test for independent samples.

    B. By comparing Kaplan-Maier survival curves (from inclusion to delivery) [log-rank test]

Ii) Multivariate analysis:

    A. A linear regression model for gestational age at birth will be performed, in which the following covariates are considered: gestational age at inclusion (continuous) and preeclampsia (binary).

    B. Survival (time between inclusion and delivery) will be analyzed by Cox regression, which considers the following covariates *: gestational age at inclusion (continuous) and preeclampsia (binary).

The number of patients included (~ 50) only allows adjustment by 2 variables to maintain a power> 80%.

Secondary objectives will be analyzed by:

I) Univariate analysis:

    A. Continuous variables: t-student (or U-Mann Whitney if non-normal distribution [Shapiro-Wilk test P <0.05)

    B. Categorical variables: 2 Pearson's (or Fisher's exact test)

Ii) Multivariate analysis using linear regression (for continuous dependent variables) and logistic regression (for binary variables)

ETHICS AND DISSEMINATION

The study will be conducted in accordance with the principles of Good Clinical Practice. This study was accepted by the Clinical Research Ethics Committee (CEIC) of Sant Joan de Déu Hospital, on July 13th 2017. Subsequent approval by individual ethical committee and competent authority was granted. Results will be published in peer-reviewed journals and disseminated at international conferences.

Patients will be informed that her participation in the trial will be treated with the same confidentiality as her clinical documentation, but, if necessary, a member of the CEIC of the center, an inspector appointed by the health authorities, or the clinical trial monitor may have access to it. In the data collection notebook, the patient will be identified only by her inclusion number in the study.

The trial will be entered in the public registry www.clinicaltrial.gov. according to Science Law 14/2011 and the results will be published in an open access journal.

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Table 1: Participant timeline

	STUDY PERIOD		
	DIAGNOSIS 24-32w	POST-ALLOCATION 24-32W	CLOSE OUT: 28 days after delivery
Eligibility screen	X		
Informed consent	X		
Allocation	X		
INTERVENTIONS			
Concealment		X	
Revealment		X	
ASSESSMENTS			
Socio-demographic data	X		
Medical history	X		
Follow-up: US biometries and Doppler assessment		X	
Ensure compliance		X	
Check side effects/ adverse events		X	
Perinatal Outcome			X

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and	2a	Scientific background and explanation of rationale	5-7
objectives	2b	Specific objectives or hypotheses	8-9
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	10
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	10
Participants	4a	Eligibility criteria for participants	10
	4b	Settings and locations where the data were collected	10
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	11
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	11-12
	6b	Any changes to trial outcomes after the trial commenced, with reasons	-----
Sample size	7a	How sample size was determined	12-13
	7b	When applicable, explanation of any interim analyses and stopping guidelines	------
Randomisation:			
Sequence	8a	Method used to generate the random allocation sequence	13
generation	8b	Type of randomisation; details of any restriction (such as blocking and block size)	13
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	13
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	13
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	13

CONSORT 2010 checklist

Page 1

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	----
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	15
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	15
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	11-12
	13b	For each group, losses and exclusions after randomisation, together with reasons	11-12
Recruitment	14a	Dates defining the periods of recruitment and follow-up	----
	14b	Why the trial ended or was stopped	----
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	---
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	---
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	---
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	---
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	---
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	---
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	4
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	---
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	---
Other information			
Registration	23	Registration number and name of trial registry	3
Protocol	24	Where the full trial protocol can be accessed, if available	3
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

CONSORT 2010 checklist

Page 2

BMJ Open

Study protocol for a pilot randomized controlled trial: Treatment of early intrauterine growth restriction with low molecular weight heparin (TRACIP)

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Manuscripts

Study protocol for a pilot randomized controlled trial: Treatment of early intrauterine growth restriction with low molecular weight heparin (TRACIP)

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ABSTRACT

Introduction: Intrauterine growth restriction (IUGR) is defined as a failure to achieve the endorsed growth potential secondary to placental insufficiency. The incidence of IUGR is estimated at about 3% of pregnancies, and it is associated with 30% of all perinatal mortality and severe morbidity with adverse neurodevelopmental and cardiovascular health consequences in adult life. Early onset IUGR represents 20–30% of all cases and is highly associated with severe placental insufficiency; subsequent chronic fetal hypoxia is the major contributor to poor perinatal outcomes. The existing evidence suggests that low molecular weight heparin (LMWH) has effects beyond its antithrombotic action, improving placental microvessel structure and function of pregnant women with vascular obstetric complications by normalizing pro-angiogenic and anti-apoptotic protein levels, cytokines, and inflammatory factors. This helps normalize placental invasion and angiogenesis activity to improve endothelial cell and trophoblast survival. The objective of our study is to demonstrate the effectiveness of low molecular weight heparin in prolonging gestation in pregnancies with early-onset IUGR. Our secondary aims are to demonstrate the effects of low molecular weight heparin on: (i) reducing neonatal morbidity, (ii) improving pro-angiogenic and anti-inflammatory maternal and placental factors, (iii) and minimizing thrombotic and ischemic placental lesions.

Methods and analysis: This is a multicenter, triple-blind, parallel-arm randomized clinical trial. Singleton pregnancies qualifying for early (20–32 weeks at diagnosis) placental IUGR (according to Delphi criteria) are those with umbilical artery Doppler with absent/reversed diastolic flow, estimated fetal weight <10th percentile plus pulsatility index (PI) of umbilical artery Doppler > 95th percentile, or estimated fetal weight <10th percentile plus mean PI of uterine artery Doppler >95th percentile. These patients will be randomized to subcutaneous (sc) treatment with bemiparin 3,500 IU/0.2 ml/day or placebo from inclusion at diagnosis to the time of delivery. Analyses will be based on originally assigned groups (intention-to-treat). The primary objective will be analyzed by comparing gestational age and prolongation of pregnancy (days) in each group with Student's t tests for independent samples and by comparing Kaplan-Maier survival curves (from inclusion to delivery, log-rank test). A linear regression model for gestational age at birth will consider the following covariates: gestational age at inclusion (continuous) and preeclampsia (binary). Survival (time between inclusion and delivery) will be analyzed by Cox regression, which considers the gestational age at inclusion (continuous) and preeclampsia (binary).

Ethics and dissemination: The study will be conducted in accordance with the principles of Good Clinical Practice. This study was approved by the Clinical Research Ethics Committee (CEIC) of Sant Joan de Déu Hospital, on July 13th 2017. The trial is registered in the public registry www.clinicaltrial.gov. according to Science Law 14/2011, and the results will be published in an open access journal.

Competing interest statement: The authors report no conflict of interest.

Trial registration number: NCT03324139

Author contributions:

EM is the project monitor

NM, AP, DO, PIB, and JS are coordinators at their respective sites.

EM is the general coordinator and PI of the project.

MC, CR, MDGR, and MDT are consultants.

FF is the co-principal investigator of the study.

STRENGTHS AND LIMITATIONS OF THIS STUDY

- This is a multicenter, triple-blinded, randomized trial with two parallel groups.
- The primary outcome (prolongation of pregnancy) is a surrogate of perinatal health.
- The sample size limits the analysis of secondary outcomes.
- Follow-up of the offspring is limited to the neonatal period.

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INTRODUCTION

BACKGROUND

Placental insufficiency and intrauterine growth restriction (IUGR)

Intrauterine growth restriction (IUGR) is defined as a failure to achieve the endorsed growth potential secondary to placental insufficiency. Its incidence is estimated at about 3% of pregnancies, and it is associated with 30% of all perinatal mortality and severe morbidity,[1] with adverse neurodevelopmental and cardiovascular health consequences in adult life.

IUGR presents as two different phenotypes depending on whether onset is early or late in gestation[1]. There is a general correspondence between early onset and the most severe forms of IUGR. Early onset IUGR represents 20–30% of all IUGR[1] and presents in association with early preeclampsia (PE) in up to 50% of cases[1]. This type of IUGR is highly associated with severe placental insufficiency; subsequent chronic fetal hypoxia is the major contributor to poor perinatal outcomes[2].

Placental perfusion depends on remodeling of maternal spiral arteries by the fetal trophoblast. Maternal spiral arteries supply blood to the placenta and fetus. To increase the blood supply up to 10 times during pregnancy, these arteries become low-resistance vessels, a process that depends on appropriate interactions between fetal trophoblast and maternal tissues[3].

The fetal cytotrophoblast modifies its epithelial phenotype by producing endothelial cells that invade maternal tissues and replacing the endothelium and the muscularis layer of maternal spiral arteries.

The key to successful placental implantation is controlled activation of the hemostatic system, which balances the process of trophoblastic invasion and protects against bleeding. In PE and early onset IUGR, this vascular remodeling does not exist or is incomplete[4], which leads to placental hypoperfusion associated with endothelial cell dysfunction and subsequent coagulation, fibrin deposition, and uteroplacental thrombosis with local implications in the case of IUGR and systemic implications in the case of PE[5]. Pathological examination of the placenta shows uteroplacental thrombosis, infarctions, and fibrin deposits.

The natural history of placental insufficiency decreases fetal well-being, with a progression to hypoxia and acidosis; in clinical practice, this deterioration is monitored by Doppler study of the umbilical artery (which reflects the placental lesion) and ductus venosus (which reflects fetal cardiac diastolic failure secondary to acidosis) to individually time the delivery in an elective and adapted manner. The latency time to severe fetal deterioration is variable but is usually 3–4 weeks[6]. It is estimated that between 24 and 28 weeks, each week of prolonged gestation increases survival

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without sequelae by 10–15%; and between 28 and 32 weeks it increases by 5%[7]. The objective of fetal monitoring is therefore to maximize gestational age at birth while minimizing the risk of death and sequelae.

Heparin and placenta: biological plausibility

There is *in vitro* evidence that heparin improves placental perfusion and stimulates neoangiogenesis. Heparin exerts its anticoagulant function through the union and potentiation of antithrombin, and it also releases tissue inhibitory factor to the circulation[4]. It also has beneficial effects through other routes that do not have to do with its anticoagulant effect[8]: it influences trophoblast growth and development, it decreases apoptosis, it acts as an indirect growth factor, and it diminishes inflammation via its anti-complement effects.

A study[9] comparing the placental microvessels of healthy pregnant women and those with vascular obstetric complications who had been treated with low molecular weight heparin demonstrated that the effects on invasion, angiogenesis, and placental cell survival were similar in these two groups. In placental microvessels from gestations with vascular complications without heparin treatment, angiogenesis and trophoblastic invasion were decreased and trophoblastic apoptosis was increased compared to tissue from healthy pregnant women[10].

Significant increases in the levels of some proteins that play important roles in placental development and angiogenesis were reported in patients treated with low molecular weight heparin, such as leptin, Ang-2, VEGFR-3, angiostatin, TIMP-1, and TNF-α. Leptin regulates the transfer of nutrients through and into the placenta[11]. Ang-2 stimulates the endothelial cell receptor Tie-2 to regulate vascular remodeling and integrity and is an important factor in placental biology and vascular development of chorionic villi. VEGFR-3 is localized in endothelial cells and syncytiotrophoblasts and is significantly diminished in cases of IUGR. Angiostatin is an endogenous inhibitor of angiogenesis that is increased in placentas obtained from women with PE. TIMP-1 is mainly found in cytotrophoblasts and the syncytiotrophoblast of the placenta and decidua. Despite decreased levels of MMP-1 in the placentas of women with PE, there are no differences between these levels of TIMP-1 and placenta levels in healthy pregnant women[12]. TNF-α participates in systemic inflammation and is released in the setting of placental ischemia/hypoxia, as well as by macrophages and systemic lymphocytes. Elevated TNF-α levels have been found in pregnancies with vascular complications[13].

In vitro results indicate that heparin normalizes MMP-9 levels at the level of placental microvessels[9]. MMP-9 controls trophoblastic invasion, and the decrease of its

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expression in trophoblasts as a response to hypoxia reduces invasiveness, which can impair placenta implantation. Low molecular weight heparin significantly increases the levels of both the pro-MMP and active forms, which increases trophoblast invasiveness and promotes angiogenesis *in vitro*.

Heparin inhibits placental apoptosis by increasing cellular protection. Levels of Bcl-2, a known inhibitor of apoptosis, were increased in an *in vitro* study of heparin treatment of cultured placenta explants, whereas Bcl-2 expression is decreased in the syncytiotrophoblasts of pregnancies complicated by growth restriction and PE[14].

Heparin therapy also normalizes levels of inflammatory factors (IFN-γ, TGF-β1, IL-6, IL-8) that are elevated in the microvessels of pregnant women with vascular complications.

Much of the available evidence comes from *in vitro* models; the clinical correlations of these effects described in experimental models are poorly characterized. These data suggest that low molecular weight heparin has effects beyond its antithrombotic action, improving the microvessel structure and function of pregnant women with vascular obstetric complications by normalizing levels of proangiogenic and antiapoptotic proteins, cytokines, and inflammatory factors at the microvessel level. These changes facilitate appropriate invasion and angiogenesis activity to promote endothelial cell and trophoblast survival.

Heparin and placenta: clinical evidence

There are few high-quality clinical studies on the efficacy of heparin in gestation. A review[15] involving 10 randomized trials with 1139 patients concluded that for women at high risk of complications secondary to placental insufficiency, treatment with heparin was associated with significant reductions in perinatal mortality, prematurity before 34 and 37 weeks, and low fetal weight for gestational age. However, these studies only include pregnant women at risk, mainly with a history of placental insufficiency in a previous pregnancy (IUGR, PE, placental abruption, or intrauterine death). Another recent meta-analysis[16] (including six clinical trials) showed that in high-risk pregnant women treated primarily with aspirin, the addition of low molecular weight heparin reduced the incidences of PE and low birth weight for gestational age by ~50%. However, another large individual patient meta-analysis failed to find any benefit of heparin in women with high-risk pregnancy[17].

Despite the evidence of potential benefit for secondary prevention of placental insufficiency and its complications, there is scant and contradictory evidence about its benefit in pregnant women who already have placental insufficiency. It would be in this clinical indication, which is associated with greater complication risk and need for

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preterm birth, that the necessary number of patients to treat may prove to be more beneficial. Four clinical trials were performed in women with placental insufficiency during gestation, and they employed heterogeneous diagnostic criteria and methodological aspects and results. Kingdom *et al.*[18] and Souza *et al.*[19] randomized patients with clinical criteria for placental insufficiency who were treated with heparin or placebo and did not observe significant differences in gestational age at birth (35.6 weeks vs 34.3 weeks and 35.69 weeks vs 33.5 weeks, respectively) or perinatal morbidity and mortality. In contrast, Yu *et al.*[20,21] demonstrated significant differences in gestational age at birth (37 weeks vs 38 weeks and 36.2 weeks vs 37.1 weeks) and fetal birth weight in patients with placental insufficiency treated with heparin.

Study justification

There is good biological evidence on the potential beneficial effects of heparin on the placenta, but the clinical correlation of the effects described in experimental models is lacking. In addition, the clinical evidence of its benefit to treat gestation already complicated with placental insufficiency is weak and contradictory. Heparin has the potential to prolong gestation during a clinically important period to maximize gestational age at birth to reduce mortality and perinatal sequelae.

HYPOTHESIS
Primary hypothesis

Treatment with low molecular weight heparin (LMWH) at the time of diagnosis of early onset IUGR prolongs the latency to severe fetal impairment, increasing the gestational age at delivery of these patients.

Secondary hypothesis

i.   Treatment with low molecular weight heparin at the time of diagnosis of early onset IUGR reduces neonatal morbidity.

ii. Treatment with low molecular weight heparin at the time of IUGR diagnosis improves maternal and placental biochemical, angiogenic, and inflammatory profiles.

iii. Treatment with low molecular weight heparin at the time of diagnosis of early-onset IUGR reduces placental lesions secondary to poor placental and fetal perfusion.

OBJECTIVES
Primary objectives

To demonstrate the effectiveness of low molecular weight heparin in prolonging gestation in pregnancies with early-onset IUGR.

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Secondary objectives

i.   To demonstrate the effectiveness of low molecular weight heparin in reducing neonatal morbidity in pregnancies with early-onset IUGR.

ii. To demonstrate the effect of low molecular weight heparin in improving pro-angiogenic and anti-inflammatory maternal and placental profiles in pregnancies with early-onset IUGR.

iii. To demonstrate the effects of low molecular weight heparin in reducing thrombotic and ischemic placental lesions.

TRIAL DESIGN

This is a multicenter, triple-blinded, randomized, phase III trial with two groups in parallel.

METHODS

STUDY SETTING

The study will be conducted within Spanish academic hospitals with specialist experience in managing IUGR: Hospital Sant Joan de Déu (Barcelona), Hospital Clínic (Barcelona), and Hospital Clínico Lozano Blesa (Zaragoza). The first two hospitals are legally integrated in the Center of Maternal Fetal and Neonatal Medicine of Barcelona (BCNatal). Each of these hospitals has over 3000 deliveries and performs more than 10,000 fetal ultrasounds annually.

ELIGIBILITY CRITERIA

Inclusion criteria:

I.   Singleton fetus.

II. Diagnosis of early onset IUGR according to the Delphi classification[22] (20–32 weeks at diagnosis) with umbilical artery Doppler with absent/reversed diastolic flow; or estimated fetal weight <10th percentile plus pulsatility index (PI) of umbilical artery Doppler >95th percentile or estimated fetal weight <10th percentile plus mean PI of uterine artery Doppler >95th percentile.

Exclusion criteria:

I.   Abnormal karyotype, structural abnormalities, or congenital infections

II. Treatment with LMWH or oral anticoagulants prior to inclusion

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III. History of heparin-induced thrombocytopenia

IV. Active hemorrhage or increased risk of bleeding due to hemostatic changes

V. Severe hepatic or pancreatic dysfunction

VI. Organic lesions that can bleed (e.g., active peptic ulcer, hemorrhagic stroke, aneurysm, or brain tumor)

Stop treatment criteria:

I. Intolerance or hypersensitivity to bemiparin

II. Allergic reaction

III. Clinically relevant bleeding (requiring hospitalization)

IV. Requirement of LMWH per clinical indication

INTERVENTION

Patients who agree to participate and provide signed informed consent signed will be centrally randomized (via a website) through the Clinical Trials Unit of Sant Joan de Déu Hospital in Barcelona (block randomization of 10) to 3,500 IU/0.2 ml/day of subcutaneous (sc) bemiparin sc or placebo of the same presentation as the active drug, from inclusion to delivery (estimated median of 5–6 weeks, maximum of 13 weeks).

The treatment will be delivered to the patient weekly using the same presentation and labeled with the study identification and participant number.

CONTROL VARIABLES:

1. Maternal age at birth; continuous (years)

2. Smoking during gestation; continuous (cigarettes/day)

3. Maternal weight at the beginning of gestation; continuous (kg)

4. Maternal height; continuous (cm)

5. Maternal ethnic origin; categorical (Europe, Africa, South America, Maghreb, Asia, Others)

6. Parity (number of deliveries >22 weeks); discrete

7. History of PE[23]; binary (Yes/No)

8. History of gestational hypertension; binary (Yes/No)

9. History of IUGR (birthweight below the 10th percentile)[24]; binary (Yes/No)

10. Gestational age at the inclusion in the study; continuous (weeks)

11. Diastolic blood pressure at inclusion; continuous (mmHg)

12. Systolic blood pressure at inclusion; continuous (mmHg)

13. Mean PI of the uterine arteries at inclusion and before delivery; continuous (normalized by gestational age)[25]

14. PI of the umbilical artery at inclusion and before delivery; continuous (normalized by gestational age)[26]

15. PI of the middle cerebral artery at inclusion and before delivery; continuous (normalized by gestational age)[27]

16. PI of the ductus venosus at inclusion and before delivery; continuous (normalized by gestational age)[28]

17. Hypertension in current pregnancy; binary (Yes/No).

18. PE in current pregnancy; binary (Yes/No).

OUTCOMES

Primary outcome

Gestational age at birth (dated by ultrasound <14 weeks by measurement of crown rump length; continuous (mm)[29]).

Secondary outcomes

1. IUGR: birthweight less than the 10th percentile for our population[24] with pulsatility index of the umbilical artery during the third trimester (on two separate occasions >48 h) higher than the 95th percentile[26]; binary (Yes/No)

2. PE[23]: diastolic blood pressure (DBP) ≥90mmHg and/or systolic (SBP) ≥140 in two separate determinations, >4 h with proteinuria >300 mg/24 h or other maternal organ dysfunction/renal insufficiency, liver involvement, neurological complications, hematological complications); binary (Yes/No)

3. Severe PE: PE with BP ≥110/160 mmHg, oliguria (<400 mL/24 h), neurological symptoms, acute pulmonary edema, persistent epigastric pain, hepatic dysfunction, analytical signs of hemolysis (lactate dehydrogenase >700 U/L), and/or thrombocytopenia (<100,000/mL); binary (Yes/No)

4. Preterm birth before 34 weeks of gestation; binary (Yes/No)

5. Emergent caesarean section for loss of fetal well-being; binary (Yes/No)

6. Birthweight; continuous (g)

7. Neonatal acidosis (arterial pH <7.10 + base excess> 12 mEq/L); binary (Yes/No)

8. Perinatal mortality (from 22 weeks of gestation to 28 days postpartum); binary (Yes/No)

9. Days in the neonatal intensive care unit; continuous (days)

10. Neonatal morbidity (seizures, intraventricular hemorrhage grade III or IV, periventricular leukomalacia, hypoxic-ischemic encephalopathy, abnormal electroencephalogram, necrotizing entercholitis, acute renal failure (serum creatinine >1.5 mg/dL) or heart failure (requiring inotropic agents); binary (Yes/No)

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11. Biomarkers in maternal blood at the time of diagnosis and delivery: sFlt and PlGF; continuous (pg/mL)

12. Biomarkers in umbilical cord blood: TNF-α, IL6, IFN-γ, FGF basic, VEGF and PlGF; continuous

13. mRNAs in trophoblast: IL6, IFN-γ, TNF-α, VEGFA, VEGFB, FGF2, and RQVEGF Receptor1; continuous

PARTICIPANT TIMELINE

Table 1 shows the participant timeline.

SAMPLE SIZE

A systematic review (PubMed and ISI Web of Knowledge) of clinical trials published in pregnant women suspected of placental insufficiency involving heparin treatment (both unfractionated and LMWH) only identified 4 studies with good or very good quality (Newcastle-Ottawa scale). The meta-analysis of these studies demonstrated very high heterogeneity under a fixed-effect model (I^2 90.4%) to estimate the difference in gestational age at birth between groups. Under a random effects model, the mean difference in gestational age at delivery was 1 week with a standard deviation of 0.9. Under these assumptions, the sample needed to detect a clinically relevant difference (fixed at 1 week) at gestational age at delivery with a power of 95% and an alpha risk of 5% was 22 patients per arm (44 patients in total). To compensate for possible withdrawals, we will require 25 per arm (50 patients).

ALLOCATION

Using an online service (http://www.randomization.com), randomization sequences will be generated in blocks of six subjects to ensure balanced distribution within study arms, stratified by participating site. Randomization sequences will be also stratified for <28 and ≥28 completed weeks and for umbilical artery Doppler with present or absent/reverse end diastolic flow. The allocation sequence will be internally sequestered by a Clinical Trials Unit (CTU).

At the time of the diagnosis and after enrollment, recruiting physicians will obtain the allocation group from a web-based system.

Each treatment pack will only be identified by a randomization code. The treatment allocation will only be revealed to the researchers after study completion or when clinically essential.

The Pharmacy Department of Sant Joan de Déu Hospital will provide labeling for all packs and blister sheets, ensuring complete blinding to all participants and

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investigators including the principal investigator, participating research doctors, project managers, and others involved in the trial.

Matching placebo will be identical to the intervention (bemiparin) in such parameters as size, physical properties, and appearance. The Pharmacy Department will keep the randomization code list confidential.

PARTICIPANT COMPLIANCE AND BLINDING PROCESS

Compliance will be assessed by trial teams by counting remaining injections at each follow-up visit and inquiring about compliance weekly.

Participants will be encouraged to report any concerns or side effects in a diary for review at each trial visit.

Blinding success will be assessed before delivery by asking the managing doctor about suspicion of treatment arm (i.e., due to the presence of bruising).

DATA COLLECTION

Patients will be recruited at the time of diagnosis of early onset IUGR, when fetal-placental Doppler study is routinely performed to obtain the mean PIs of the uterine arteries, umbilical artery, middle cerebral artery, and ductus venosus .

Maternal blood will be collected at the time of diagnosis to study angiogenic factors.

The control variables defined in the previous section will also be collected in the first visit. Participant data from this first visit and subsequent visits for this study (anonymized) will be entered into an electronic case report form (e-CRF) hosted on a secure website by each site coordinator. Logic and range rules operate in the e-CRF to minimize imputation errors.

Maternal blood, cord blood, and placenta will be collected at the time of delivery. Perinatal outcomes will be collected after delivery.

Obtaining and processing of maternal blood samples:

We will collect 5 mL maternal blood in tubes without anticoagulant. They will be transported to the laboratory within 1 hour after extraction, maintaining each center's safety guidelines for the transport of biological material. Blood tubes will be centrifuged at 1500 g for 15 minutes. Supernatant (serum) will be aspirated, stored in cryovials, properly labeled, and kept in a –80°C freezer. Levels of PlGF and SFlt-1 (pg/mL) will be determined using an Elecsys automated platform by electrochemiluminescence (Cobas analyzers, Roche Diagnostics). The intra- and inter-assay coefficients of variation are <4% and 2.4 to 4.6%, respectively.

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Collection and processing of cord blood samples:

After fetal extraction, umbilical cord venous blood will be collected in a 10-mL silicone tube without additives. Samples will be transported to the laboratory within 1 hour of extraction, maintaining our center's safety guidelines for transport of biological material. Blood tubes will be centrifuged at 1500 g for 15 minutes. Supernatant (serum) will be aspirated and stored in properly labeled cryovials in an -80ºC freezer until analysis in duplicate in the LABSCAN 100 Multiplex Analyzer (Luminex) (LUMINEX 100 IS software).

Collection and processing of placental tissue samples:

After delivery, two placental tissue biopsies will be stored in later RNA in aliquots at –80ºC until the time of analysis. DNA will be extracted from fresh placental tissue with Flexigene (Qiagen). Placentas will then be fixed in buffered formalin. After gross examination, routine samples will be obtained from each specimen: one transverse section of cord, one rolled strip of membranes, and three blocks from the placental parenchyma. Additional blocks will be taken from all macroscopic lesions. Placental findings will be reported as described in the Amsterdam Consensus[30].

Maternal blood, cord blood, and placental tissue samples will be analyzed in the *Centro de Investigación Biomédica de Aragón (CIBA), Aragon Institute for Health Research (IIS Aragón) Zaragoza, Spain*.

DATA MONITORING AND SAFETY

An independent Clinical Trial Unit will perform an offline data audit every 6 months checking for missing information and errors. The same committee will monitor safety issues. There may be unexpected adverse reactions associated with low molecular weight heparin. All investigators have a thorough understanding of the anticipated adverse events and the appropriate reporting process. The investigators will notify the Independent Clinical Trial Unit of adverse events, and they will report to the regulatory authority and ethics committee.

STATISTICAL ANALYSIS

Analysis will be based on originally assigned groups (intention-to-treat). However, it is possible that there will still be some missing data at the end of the study, so sensitivity analyses will be carried out to confirm the robustness of the results. Methods based around multiple imputation will be used.

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The primary objective will be analyzed by:

I) Univariate analysis:

A. Compare gestational age and prolongation of pregnancy (days) in each group using Student's t tests for independent samples

B. Compare Kaplan-Maier survival curves (from inclusion to delivery, log-rank test)

II) Multivariate analysis:

A. A linear regression model for gestational age at birth will be performed, in which the following covariates are considered: gestational age at inclusion (continuous) and PE (binary).

B. Survival (time between inclusion and delivery) will be analyzed by Cox regression considering the following covariates *: gestational age at inclusion (continuous) and PE (binary).

The number of patients included (~50) only allows adjustment by 2 variables to maintain power >80%.

Secondary objectives will be analyzed by:

I) Univariate analysis:

A. Continuous variables: Student's t test (or Mann-Whitney U tests for non-normal distribution, Shapiro-Wilk's test P<0.05)

B. Categorical variables: 2 Pearson's (or Fisher's exact test)

II) Multivariate analysis using linear regression (for continuous dependent variables) or logistic regression (for binary variables)

ETHICS AND DISSEMINATION

The study will be conducted in accordance with the principles of Good Clinical Practice. This protocol was approved by the Clinical Research Ethics Committee (CEIC) of Sant Joan de Déu Hospital on July 13th 2017. Subsequent approval by individual ethical committees and competent authority was granted. Results will be published in peer-reviewed journals and disseminated at international conferences.

Patients will be informed that her participation in the trial will be treated with the same confidentiality as their clinical documentation, but, if necessary, a member of the CEIC of the center, an inspector appointed by the health authorities, or the clinical trial monitor may have access to it. In the data collection notebook, the patient will only be identified by her study inclusion number.

The trial has been entered in the public registry www.clinicaltrial.gov (trial registration number NCT03324139) according to Science Law 14/2011, and the results will be published in an open access journal.

DISCUSSION

The main limitation is that it this is a pilot study with a small sample size. However, the results will be very useful for designing larger, future trials, and it will provide mechanistic data on biomarkers in maternal blood, cord blood, and placenta.

Although prolongation of pregnancy is a useful surrogate of perinatal health, the study would be largely underpowered if we set neonatal mortality/morbidity as a primary outcome.

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Table 1: Participant timeline

		STUDY PERIOD		
	DIAGNOSIS 20–32 w	POST-ALLOCATION 24-32 w	DELIVERY	CLOSE OUT: 28 d after delivery
Eligibility screen	X			
Informed consent	X			
Allocation	X			
INTERVENTIONS				
Concealment		X		
Revealment		X		
ASSESSMENTS				
Socio-demographic data	X			
Medical history	X			
Follow-up: ultrasound biometries and Doppler assessment		X[1]		
Biomarkers in maternal blood	X		X	
Ensure compliance		X		
Biomarkers in cord blood and placenta			X	
Check side effects/ adverse events		X		
Perinatal outcome				X

[1]According to local protocol

(https://medicinafetalbarcelona.org/clinica/images/protocolos/patologia__fetal/cir-peg.pdf)

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SPIRIT☑

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____1_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	_____3_____
	2b	All items from the World Health Organization Trial Registration Data Set	__------____
Protocol version	3	Date and version identifier	__--------_____
Funding	4	Sources and types of financial, material, and other support	_____3_____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1 and 3
	5b	Name and contact information for the trial sponsor	____-----_____
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	__------___
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	_____--------_____

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3   **Introduction**
4
5   Background and   6a   Description of research question and justification for undertaking the trial, including summary of relevant   _5-8_____
6   rationale   studies (published and unpublished) examining benefits and harms for each intervention
7
8   6b   Explanation for choice of comparators   5-8_____
9
10   Objectives   7   Specific objectives or hypotheses   _____8-9_____
11
12   Trial design   8   Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group),
13   allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)   ___9_____
14
15   **Methods: Participants, interventions, and outcomes**
16
17   Study setting   9   Description of study settings (eg, community clinic, academic hospital) and list of countries where data will _____9_____
18   be collected. Reference to where list of study sites can be obtained
19
20   Eligibility criteria   10   Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and   ____9-10_____
21   individuals who will perform the interventions (eg, surgeons, psychotherapists)
22
23   Interventions   11a   Interventions for each group with sufficient detail to allow replication, including how and when they will be   ___10_____
24   administered
25
26   11b   Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose   _10_____
27   change in response to harms, participant request, or improving/worsening disease)
28
29   11c   Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence   _13_____
30   (eg, drug tablet return, laboratory tests)
31
32   11d   Relevant concomitant care and interventions that are permitted or prohibited during the trial   _10_____
33
34   Outcomes   12   Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood
35   pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, 11-12__
36   median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen
37   efficacy and harm outcomes is strongly recommended
38
39   Participant timeline   13   Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for 12 and 20___
40   participants. A schematic diagram is highly recommended (see Figure)
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3 Sample size   14   Estimated number of participants needed to achieve study objectives and how it was determined, including   12_____
4   clinical and statistical assumptions supporting any sample size calculations
5
6 Recruitment   15   Strategies for achieving adequate participant enrolment to reach target sample size   __12_____
7

Methods: Assignment of interventions (for controlled trials)

Allocation:

Sequence   16a   Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any   12_____
generation   factors for stratification. To reduce predictability of a random sequence, details of any planned restriction
                       (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants
                       or assign interventions

Allocation   16b   Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered,   __12_____
concealment   opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned
mechanism

Implementation   16c   Who will generate the allocation sequence, who will enrol participants, and who will assign participants to   ____12_____
                       interventions

Blinding (masking)   17a   Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome   ___12 and 13____
                       assessors, data analysts), and how

                 17b   If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's   ___10_____
                       allocated intervention during the trial

Methods: Data collection, management, and analysis

Data collection   18a   Plans for assessment and collection of outcome, baseline, and other trial data, including any related   __13-14_____
methods   processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of
                       study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known.
                       Reference to where data collection forms can be found, if not in the protocol

                 18b   Plans to promote participant retention and complete follow-up, including list of any outcome data to be   ___13-14_____
                       collected for participants who discontinue or deviate from intervention protocols

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3 Data management 19 Plans for data entry, coding, security, and storage, including any related processes to promote data quality 13_____
4 (eg, double data entry; range checks for data values). Reference to where details of data management
5 procedures can be found, if not in the protocol
6
7 Statistical methods 20a Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the __14-15_____
8 statistical analysis plan can be found, if not in the protocol
9
10 20b Methods for any additional analyses (eg, subgroup and adjusted analyses) 14-15__
11
12 20c Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any
13 statistical methods to handle missing data (eg, multiple imputation) 14-15_____
14
15 **Methods: Monitoring**
16
17 Data monitoring 21a Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of ____14_____
18 whether it is independent from the sponsor and competing interests; and reference to where further details
19 about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not
20 needed
21
22 21b Description of any interim analyses and stopping guidelines, including who will have access to these interim _____14_____
23 results and make the final decision to terminate the trial
24
25 Harms 22 Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse _____14_____
26 events and other unintended effects of trial interventions or trial conduct
27
28 Auditing 23 Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent ____14_____
29 from investigators and the sponsor
30
31
32 **Ethics and dissemination**
33
34 Research ethics 24 Plans for seeking research ethics committee/institutional review board (REC/IRB) approval ___15-16_____
35 approval
36
37 Protocol 25 Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, _____15-16
38 amendments analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals,
39 regulators)
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Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	10_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	--------_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	15_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	3_____
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	15-16_____
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	---------_____
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	15-16_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	----------_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	---------_____

Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	---------_____
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	_------------_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

5

Study protocol for a randomized controlled trial: Treatment of early intrauterine growth restriction with low molecular weight heparin (TRACIP)

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-020501.R2
Article Type:	Protocol
Date Submitted by the Author:	13-Mar-2018
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Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Obstetrics and gynaecology
Keywords:	Fetal medicine < OBSTETRICS, Prenatal diagnosis < OBSTETRICS, Ultrasonography < OBSTETRICS

SCHOLARONE™
Manuscripts

Study protocol for a randomized controlled trial: Treatment of early intrauterine growth restriction with low molecular weight heparin (TRACIP)

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ABSTRACT

Introduction: The incidence of Intrauterine growth restriction (IUGR) is estimated at about 3% of pregnancies, and it is associated with 30% of all perinatal mortality and severe morbidity with adverse neurodevelopmental and cardiovascular health consequences in adult life. Early onset IUGR represents 20–30% of all cases and is highly associated with severe placental insufficiency. The existing evidence suggests that low molecular weight heparin (LMWH) has effects beyond its antithrombotic action, improving placental microvessel structure and function of pregnant women with vascular obstetric complications by normalizing pro-angiogenic and anti-apoptotic protein levels, cytokines, and inflammatory factors. The objective of our study is to demonstrate the effectiveness of low molecular weight heparin in prolonging gestation in pregnancies with early-onset IUGR.

Methods and analysis: This is a multicenter, triple-blind, parallel-arm randomized clinical trial. Singleton pregnancies qualifying for early (20–32 weeks at diagnosis) placental IUGR (according to Delphi criteria) will be randomized to subcutaneous (sc) treatment with bemiparin 3,500 IU/0.2 ml/day or placebo from inclusion at diagnosis to the time of delivery. Analyses will be based on originally assigned groups (intention-to-treat). The primary objective will be analyzed by comparing gestational age and prolongation of pregnancy (days) in each group with Student's t tests for independent samples and by comparing Kaplan-Maier survival curves (from inclusion to delivery, log-rank test). A linear regression model for gestational age at birth will consider the following covariates: gestational age at inclusion (continuous) and preeclampsia (binary).

Ethics and dissemination: The study will be conducted in accordance with the principles of Good Clinical Practice. This study was approved by the Clinical Research Ethics Committee (CEIC) of Sant Joan de Déu Hospital, on July 13th 2017. The trial is registered in the public registry www.clinicaltrial.gov. according to Science Law 14/2011, and the results will be published in an open access journal.

Competing interest statement: The authors report no conflict of interest.

Trial registration number: NCT03324139

Author contributions:

EM is the project monitor

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NM, AP, DO, PIB, and JS are coordinators at their respective sites.

EM is the general coordinator and PI of the project.

MC, CR, MDGR, and MDT are consultants.

FF is the co-principal investigator of the study.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- This is a multicenter, triple-blinded, randomized trial with two parallel groups.
- The primary outcome (prolongation of pregnancy) is a surrogate of perinatal health.
- The sample size limits the analysis of secondary outcomes.
- Follow-up of the offspring is limited to the neonatal period.

4

INTRODUCTION

BACKGROUND

Placental insufficiency and intrauterine growth restriction (IUGR)

Intrauterine growth restriction (IUGR) is defined as a failure to achieve the endorsed growth potential secondary to placental insufficiency. Its incidence is estimated at about 3% of pregnancies, and it is associated with 30% of all perinatal mortality and severe morbidity,[1] with adverse neurodevelopmental and cardiovascular health consequences in adult life.

Early onset IUGR represents 20–30% of all IUGR[1] and presents in association with early preeclampsia (PE) in up to 50% of cases[1]. This type of IUGR is highly associated with severe placental insufficiency; subsequent chronic fetal hypoxia is the major contributor to poor perinatal outcomes[2].

The key to successful placental implantation is controlled activation of the hemostatic system, which balances the process of trophoblastic invasion and protects against bleeding[3]. In PE and early onset IUGR, this vascular remodeling does not exist or is incomplete[4], which leads to placental hypoperfusion associated with endothelial cell dysfunction and subsequent coagulation, fibrin deposition, and uteroplacental thrombosis with local implications in the case of IUGR and systemic implications in the case of PE[5]. Pathological examination of the placenta shows uteroplacental thrombosis, infarctions, and fibrin deposits.

The natural history of placental insufficiency decreases fetal well-being, with a progression to hypoxia and acidosis; in clinical practice, this deterioration is monitored by Doppler study of the umbilical artery (which reflects the placental lesion) and ductus venosus (which reflects fetal cardiac diastolic failure secondary to acidosis) to individually time the delivery in an elective and adapted manner. The latency time to severe fetal deterioration is variable but is usually 3–4 weeks[6]. It is estimated that between 24 and 28 weeks, each week of prolonged gestation increases survival without sequelae by 10–15%; and between 28 and 32 weeks it increases by 5%[7]. The objective of fetal monitoring is therefore to maximize gestational age at birth while minimizing the risk of death and sequelae.

Heparin and placenta: biological plausibility

There is *in vitro* evidence that heparin improves placental perfusion and stimulates neoangiogenesis. Heparin exerts its anticoagulant function through the union and potentiation of antithrombin, and it also releases tissue inhibitory factor to the circulation[4]. It also has beneficial effects through other routes that do not have to do with its anticoagulant effect[8]: it influences trophoblast growth and development, it

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decreases apoptosis, it acts as an indirect growth factor, and it diminishes inflammation via its anti-complement effects[9,10].

Significant increases in the levels of some proteins that play important roles in placental development and angiogenesis were reported in patients treated with low molecular weight heparin, such as leptin, Ang-2, VEGFR-3, angiostatin, TIMP-1, and TNF-α[11-13]

In vitro results indicate that heparin normalizes MMP-9 levels at the level of placental microvessels[9]. MMP-9 controls trophoblastic invasion, and the decrease of its expression in trophoblasts as a response to hypoxia reduces invasiveness, which can impair placenta implantation. Low molecular weight heparin significantly increases the levels of both the pro-MMP and active forms, which increases trophoblast invasiveness and promotes angiogenesis *in vitro*.

Heparin also inhibits placental apoptosis by increasing cellular protection[14] and normalizes levels of inflammatory factors (IFN-γ, TGF-β1, IL-6, IL-8) that are elevated in the microvessels of pregnant women with vascular complications.

Much of the available evidence comes from *in vitro* models; the clinical correlations of these effects described in experimental models are poorly characterized. These data suggest that low molecular weight heparin has effects beyond its antithrombotic action, improving the microvessel structure and function of pregnant women with vascular obstetric complications by normalizing levels of proangiogenic and antiapoptotic proteins, cytokines, and inflammatory factors at the microvessel level. These changes facilitate appropriate invasion and angiogenesis activity to promote endothelial cell and trophoblast survival.

Heparin and placenta: clinical evidence

There are few high-quality clinical studies on the efficacy of heparin in gestation. A review[15] involving 10 randomized trials with 1139 patients concluded that for women at high risk of complications secondary to placental insufficiency, treatment with heparin was associated with significant reductions in perinatal mortality, prematurity before 34 and 37 weeks, and low fetal weight for gestational age. However, these studies only include pregnant women at risk, mainly with a history of placental insufficiency in a previous pregnancy. Another recent meta-analysis[16] (including six clinical trials) showed that in high-risk pregnant women treated primarily with aspirin, the addition of low molecular weight heparin reduced the incidences of PE and low birth weight for gestational age by ~50%. However, another large individual patient meta-analysis failed to find any benefit of heparin in women with high-risk pregnancy[17].

Despite the evidence of potential benefit for secondary prevention of placental insufficiency and its complications, there is scant and contradictory evidence about its benefit in pregnant women who already have placental insufficiency. It would be in this clinical indication, which is associated with greater complication risk and need for preterm birth, that the necessary number of patients to treat may prove to be more beneficial. Four clinical trials were performed in women with placental insufficiency during gestation, and they employed heterogeneous diagnostic criteria and methodological aspects and results. Kingdom *et al*.[18] and Souza *et al*.[19] randomized patients with clinical criteria for placental insufficiency who were treated with heparin or placebo and did not observe significant differences in gestational age at birth (35.6 weeks vs 34.3 weeks and 35.69 weeks vs 33.5 weeks, respectively) or perinatal morbidity and mortality. In contrast, Yu *et al*.[20,21] demonstrated significant differences in gestational age at birth (37 weeks vs 38 weeks and 36.2 weeks vs 37.1 weeks) and fetal birth weight in patients with placental insufficiency treated with heparin.

Study justification

There is good biological evidence on the potential beneficial effects of heparin on the placenta, but the clinical correlation of the effects described in experimental models is lacking. In addition, the clinical evidence of its benefit to treat gestation already complicated with placental insufficiency is weak and contradictory. Heparin has the potential to prolong gestation during a clinically important period to maximize gestational age at birth to reduce mortality and perinatal sequelae.

HYPOTHESIS
Primary hypothesis

Treatment with low molecular weight heparin (LMWH) at the time of diagnosis of early onset IUGR prolongs the latency to severe fetal impairment, increasing the gestational age at delivery of these patients.

Secondary hypothesis

i.   Treatment with low molecular weight heparin at the time of diagnosis of early onset IUGR reduces neonatal morbidity.

ii. Treatment with low molecular weight heparin at the time of IUGR diagnosis improves maternal and placental biochemical, angiogenic, and inflammatory profiles.

iii. Treatment with low molecular weight heparin at the time of diagnosis of early-onset IUGR reduces placental lesions secondary to poor placental and fetal perfusion.

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OBJECTIVES

Primary objectives

To demonstrate the effectiveness of low molecular weight heparin in prolonging gestation in pregnancies with early-onset IUGR.

Secondary objectives

i. To demonstrate the effectiveness of low molecular weight heparin in reducing neonatal morbidity in pregnancies with early-onset IUGR.

ii. To demonstrate the effect of low molecular weight heparin in improving pro-angiogenic and anti-inflammatory maternal and placental profiles in pregnancies with early-onset IUGR.

iii. To demonstrate the effects of low molecular weight heparin in reducing thrombotic and ischemic placental lesions.

TRIAL DESIGN

This is a multicenter, triple-blinded, randomized, phase III trial with two groups in parallel.

METHODS

STUDY SETTING

The study will be conducted within Spanish academic hospitals with specialist experience in managing IUGR: Hospital Sant Joan de Déu (Barcelona), Hospital Clínic (Barcelona), and Hospital Clínico Lozano Blesa (Zaragoza). The first two hospitals are legally integrated in the Center of Maternal Fetal and Neonatal Medicine of Barcelona (BCNatal). Each of these hospitals has over 3000 deliveries and performs more than 10,000 fetal ultrasounds annually.

ELIGIBILITY CRITERIA

Inclusion criteria:

I. Singleton fetus.

II. Diagnosis of early onset IUGR according to the Delphi classification[22] (20–32 weeks at diagnosis) with umbilical artery Doppler with absent/reversed diastolic flow; or estimated fetal weight <10th percentile plus pulsatility index (PI) of

umbilical artery Doppler >95th percentile or estimated fetal weight <10th percentile plus mean PI of uterine artery Doppler >95th percentile.

The weeks for inclusion will be between 20-32 weeks of gestation, at the moment of the diagnosis.

Exclusion criteria:

I. Abnormal karyotype, structural abnormalities, or congenital infections
II. Treatment with LMWH or oral anticoagulants prior to inclusion
III. History of heparin-induced thrombocytopenia
IV. Active hemorrhage or increased risk of bleeding due to hemostatic changes
V. Severe hepatic or pancreatic dysfunction
VI. Organic lesions that can bleed (e.g., active peptic ulcer, hemorrhagic stroke, aneurysm, or brain tumor)

Stop treatment criteria:

I. Intolerance or hypersensitivity to bemiparin
II. Allergic reaction
III. Clinically relevant bleeding (requiring hospitalization)
IV. Requirement of LMWH per clinical indication

INTERVENTION

Patients who agree to participate and provide signed informed consent signed will be centrally randomized (via a website) through the Clinical Trials Unit of Sant Joan de Déu Hospital in Barcelona (block randomization of 10) to 3,500 IU/0.2 ml/day of subcutaneous (sc) bemiparin sc or placebo of the same presentation as the active drug, from inclusion to delivery (estimated median of 5–6 weeks, maximum of 17 weeks).

The treatment will be delivered to the patient weekly using the same presentation and labeled with the study identification and participant number.

CONTROL VARIABLES:

1. Maternal age at birth; continuous (years)
2. Smoking during gestation; continuous (cigarettes/day)
3. Maternal weight at the beginning of gestation; continuous (kg)
4. Maternal height; continuous (cm)
5. Maternal ethnic origin; categorical (Europe, Africa, South America, Maghreb, Asia, Others)
6. Parity (number of deliveries >22 weeks); discrete
7. History of PE[23]; binary (Yes/No)

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8. History of gestational hypertension; binary (Yes/No)

9. History of IUGR (birthweight below the 10th percentile)[24]; binary (Yes/No)

10. Gestational age at the inclusion in the study; continuous (weeks)

11. Diastolic blood pressure at inclusion; continuous (mmHg)

12. Systolic blood pressure at inclusion; continuous (mmHg)

13. Mean PI of the uterine arteries at inclusion and before delivery; continuous (normalized by gestational age)[25]

14. PI of the umbilical artery at inclusion and before delivery; continuous (normalized by gestational age)[26]

15. PI of the middle cerebral artery at inclusion and before delivery; continuous (normalized by gestational age)[27]

16. PI of the ductus venosus at inclusion and before delivery; continuous (normalized by gestational age)[28]

17. Hypertension in current pregnancy; binary (Yes/No).

18. PE in current pregnancy; binary (Yes/No).

OUTCOMES

Primary outcomes

Gestational age at live birth (dated by ultrasound <14 weeks by measurement of crown rump length; continuous (mm)[29]).

Prolongation of pregnancy: time from inclusion to live birth.

Secondary outcomes

1. IUGR: birthweight less than the 10th percentile for our population[24] with pulsatility index of the umbilical artery during the third trimester (on two separate occasions >48 h) higher than the 95th percentile[26]; binary (Yes/No)

2. PE[23]: diastolic blood pressure (DBP) ≥90mmHg and/or systolic (SBP) ≥140 in two separate determinations, >4 h with proteinuria >300 mg/24 h or other maternal organ dysfunction/renal insufficiency, liver involvement, neurological complications, hematological complications); binary (Yes/No)

3. Severe PE: PE with BP ≥110/160 mmHg, oliguria (<400 mL/24 h), neurological symptoms, acute pulmonary edema, persistent epigastric pain, hepatic dysfunction, analytical signs of hemolysis (lactate dehydrogenase >700 U/L), and/or thrombocytopenia (<100,000/mL); binary (Yes/No)

4. Preterm birth before 34 weeks of gestation; binary (Yes/No)

5. Emergent caesarean section for loss of fetal well-being; binary (Yes/No)

6. Birthweight; continuous (g)

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7. Neonatal acidosis (arterial pH <7.10 + base excess> 12 mEq/L); binary (Yes/No)

8. Perinatal mortality (from 22 weeks of gestation to 28 days postpartum); binary (Yes/No)

9. Days in the neonatal intensive care unit; continuous (days)

10. Neonatal morbidity (seizures, intraventricular hemorrhage grade III or IV, periventricular leukomalacia, hypoxic-ischemic encephalopathy, abnormal electroencephalogram, necrotizing entercholitis, acute renal failure (serum creatinine >1.5 mg/dL) or heart failure (requiring inotropic agents); binary (Yes/No)

11. Biomarkers in maternal blood at the time of diagnosis and delivery: sFlt and PlGF; continuous (pg/mL)

12. Biomarkers in umbilical cord blood: TNF-α, IL6, IFN-γ, FGF basic, VEGF and PlGF; continuous

13. mRNAs in trophoblast: IL6, IFN-γ, TNF-α, VEGFA, VEGFB, FGF2, and RQVEGF Receptor1; continuous

PARTICIPANT TIMELINE

Table 1 shows the participant timeline.

SAMPLE SIZE

A systematic review (PubMed and ISI Web of Knowledge) of clinical trials published in pregnant women suspected of placental insufficiency involving heparin treatment (both unfractionated and LMWH) only identified 4 studies with good or very good quality (Newcastle-Ottawa scale). The meta-analysis of these studies demonstrated very high heterogeneity under a fixed-effect model (I^2 90.4%) to estimate the difference in gestational age at birth between groups. Under a random effects model, the mean difference in gestational age at delivery was 1 week with a standard deviation of 0.9. Under these assumptions, the sample needed to detect a clinically relevant difference (fixed at 1 week) at gestational age at delivery with a power of 95% and an alpha risk of 5% was 22 patients per arm (44 patients in total). To compensate for possible withdrawals, we will require 25 per arm (50 patients).

ALLOCATION

Using an online service (http://www.randomization.com), randomization sequences will be generated in blocks of six subjects to ensure balanced distribution within study arms, stratified by participating site. Randomization sequences will be also stratified for <28 and ≥28 completed weeks and for umbilical artery Doppler with present or

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absent/reverse end diastolic flow. The allocation sequence will be internally sequestered by a Clinical Trials Unit (CTU).

At the time of the diagnosis and after enrollment, recruiting physicians will obtain the allocation group from a web-based system.

Each treatment pack will only be identified by a randomization code. The treatment allocation will only be revealed to the researchers after study completion or when clinically essential.

The Pharmacy Department of Sant Joan de Déu Hospital will provide labeling for all packs and blister sheets, ensuring complete blinding to all participants and investigators including the principal investigator, participating research doctors, project managers, and others involved in the trial.

Matching placebo will be identical to the intervention (bemiparin) in such parameters as size, physical properties, and appearance. The Pharmacy Department will keep the randomization code list confidential.

PARTICIPANT COMPLIANCE AND BLINDING PROCESS

Compliance will be assessed by trial teams by counting remaining injections at each follow-up visit and inquiring about compliance weekly.

Participants will be encouraged to report any concerns or side effects in a diary for review at each trial visit.

Blinding success will be assessed before delivery by asking the managing doctor about suspicion of treatment arm (i.e., due to the presence of bruising).

DATA COLLECTION

Patients will be recruited at the time of diagnosis of early onset IUGR, when fetal-placental Doppler study is routinely performed to obtain the mean PIs of the uterine arteries, umbilical artery, middle cerebral artery, and ductus venosus .

Maternal blood will be collected at the time of diagnosis to study angiogenic factors.

The control variables defined in the previous section will also be collected in the first visit. Participant data from this first visit and subsequent visits for this study (anonymized) will be entered into an electronic case report form (e-CRF) hosted on a secure website by each site coordinator. Logic and range rules operate in the e-CRF to minimize imputation errors.

Maternal blood, cord blood, and placenta will be collected at the time of delivery. Perinatal outcomes will be collected after delivery.

Obtaining and processing of maternal blood samples:

We will collect 5 mL maternal blood in tubes without anticoagulant. They will be transported to the laboratory within 1 hour after extraction, maintaining each center's safety guidelines for the transport of biological material. Blood tubes will be centrifuged at 1500 g for 15 minutes. Supernatant (serum) will be aspirated, stored in cryovials, properly labeled, and kept in a –80ºC freezer. Levels of PlGF and SFlt-1 (pg/mL) will be determined using an Elecsys automated platform by electrochemiluminescence (Cobas analyzers, Roche Diagnostics). The intra- and inter-assay coefficients of variation are <4% and 2.4 to 4.6%, respectively.

Collection and processing of cord blood samples:

After fetal extraction, umbilical cord venous blood will be collected in a 10-mL silicone tube without additives. Samples will be transported to the laboratory within 1 hour of extraction, maintaining our center's safety guidelines for transport of biological material. Blood tubes will be centrifuged at 1500 g for 15 minutes. Supernatant (serum) will be aspirated and stored in properly labeled cryovials in an -80ºC freezer until analysis in duplicate in the LABSCAN 100 Multiplex Analyzer (Luminex) (LUMINEX 100 IS software).

Collection and processing of placental tissue samples:

After delivery, two placental tissue biopsies will be stored in later RNA in aliquots at –80ºC until the time of analysis. DNA will be extracted from fresh placental tissue with Flexigene (Qiagen). Placentas will then be fixed in buffered formalin. After gross examination, routine samples will be obtained from each specimen: one transverse section of cord, one rolled strip of membranes, and three blocks from the placental parenchyma. Additional blocks will be taken from all macroscopic lesions. Placental findings will be reported as described in the Amsterdam Consensus[30].

Maternal blood, cord blood, and placental tissue samples will be analyzed in the *Centro de Investigación Biomédica de Aragón (CIBA), Aragon Institute for Health Research (IIS Aragón) Zaragoza, Spain.*

DATA MONITORING AND SAFETY

An independent Clinical Trial Unit will perform an offline data audit every 6 months checking for missing information and errors. The same committee will monitor safety issues. There may be unexpected adverse reactions associated with low molecular

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weight heparin. All investigators have a thorough understanding of the anticipated adverse events and the appropriate reporting process. The investigators will notify the Independent Clinical Trial Unit of adverse events, and they will report to the regulatory authority and ethics committee.

STATISTICAL ANALYSIS

Analysis will be based on originally assigned groups (intention-to-treat). However, it is possible that there will still be some missing data at the end of the study, so sensitivity analyses will be carried out to confirm the robustness of the results.

The primary objectives will be analyzed by linear regression model for gestational age and time from randomisation to live birth, in which the following covariates are considered: gestational age at inclusion (continuous) and PE (binary). Stillbirths will be considered as Missing data will be addressed by multiple imputation methods. Stillbirth cases will be penalized in the analysis by imputing them 0 days of prolongation.

The number of patients included (~50) only allows adjustment by 2 variables to maintain power >80%.

Secondary objectives will be analyzed by:

I) Univariate analysis:

      A. Continuous variables: Student's t test (or Mann-Whitney U tests for non-normal distribution, Shapiro-Wilk's test $P<0.05$)

      B. Categorical variables: 2 Pearson's (or Fisher's exact test)

II) Multivariate analysis using linear regression (for continuous dependent variables) or logistic regression (for binary variables).

ETHICS AND DISSEMINATION

The study will be conducted in accordance with the principles of Good Clinical Practice. This protocol was approved by the Clinical Research Ethics Committee (CEIC) of Sant Joan de Déu Hospital on July 13th 2017. Subsequent approval by individual ethical committees and competent authority was granted. Results will be published in peer-reviewed journals and disseminated at international conferences.

Patients will be informed that her participation in the trial will be treated with the same confidentiality as their clinical documentation, but, if necessary, a member of the CEIC of the center, an inspector appointed by the health authorities, or the clinical trial monitor may have access to it. In the data collection notebook, the patient will only be identified by her study inclusion number.

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The trial has been entered in the public registry www.clinicaltrial.gov (trial registration number NCT03324139) according to Science Law 14/2011, and the results will be published in an open access journal.

DISCUSSION

The main limitation is that it this is a pilot study with a small sample size. However, the results will be very useful for designing larger, future trials, and it will provide mechanistic data on biomarkers in maternal blood, cord blood, and placenta.

Although prolongation of pregnancy is a useful surrogate of perinatal health, the study would be largely underpowered if we set neonatal mortality/morbidity as a primary outcome.

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Table 1: Participant timeline

	DIAGNOSIS 20–32 w	STUDY PERIOD		
		POST-ALLOCATION 20-32 w	DELIVERY	CLOSE OUT: 28 d after delivery
Eligibility screen	X			
Informed consent	X			
Allocation	X			
INTERVENTIONS				
Concealment		X		
Revealment		X		
ASSESSMENTS				
Socio-demographic data	X			
Medical history	X			
Follow-up: ultrasound biometries and Doppler assessment		X[1]		
Biomarkers in maternal blood	X		X	
Ensure compliance		X		
Biomarkers in cord blood and placenta			X	
Check side effects/ adverse events		X		
Perinatal outcome				X

[1]According to local protocol

(https://medicinafetalbarcelona.org/clinica/images/protocolos/patologia__fetal/cir-peg.pdf)

SPIRIT ✓

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____1_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	_____3_____
	2b	All items from the World Health Organization Trial Registration Data Set	__------____
Protocol version	3	Date and version identifier	__--------_____
Funding	4	Sources and types of financial, material, and other support	_____3_____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1 and 3
	5b	Name and contact information for the trial sponsor	____-----_____
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	__------___
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	_____--------_____

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Introduction

| Background and rationale | 6a | Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention | _5-8_____ |

| | 6b | Explanation for choice of comparators | 5-8_____ |

| Objectives | 7 | Specific objectives or hypotheses | _____8-9_____ |

| Trial design | 8 | Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) | ___9_____ |

Methods: Participants, interventions, and outcomes

| Study setting | 9 | Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained | _____9_____ |

| Eligibility criteria | 10 | Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) | ____9-10_____ |

| Interventions | 11a | Interventions for each group with sufficient detail to allow replication, including how and when they will be administered | ___10_____ |

| | 11b | Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) | _10_____ |

| | 11c | Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) | _13_____ |

| | 11d | Relevant concomitant care and interventions that are permitted or prohibited during the trial | _10_____ |

| Outcomes | 12 | Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended | 11-12__ |

| Participant timeline | 13 | Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) | 12 and 20___ |

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Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations

12_____

Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size

__12_____

Methods: Assignment of interventions (for controlled trials)

Allocation:

Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions

12_____

Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned

__12_____

Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions

____12_____

Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how

___12 and 13____

	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial

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Methods: Data collection, management, and analysis

Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol

__13-14_____

	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols

___13-14_____

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Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	13_____
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	__14-15_____
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	14-15__
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	14-15_____

Methods: Monitoring

Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	____14_____
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	_____14_____
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	_____14_____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	____14_____

Ethics and dissemination

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	___15-16_____
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	_____15-16

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Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	10_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	--------_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	15_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	3_____
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	15-16_____
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	---------_____
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	15-16_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	----------_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	---------_____

Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	---------_____
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	_------------_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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BMJ Open

Study protocol for a randomized controlled trial: Treatment of early intrauterine growth restriction with low molecular weight heparin (TRACIP)

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-020501.R3
Article Type:	Protocol
Date Submitted by the Author:	07-May-2018
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Primary Subject Heading:	Obstetrics and gynaecology
Secondary Subject Heading:	Obstetrics and gynaecology
Keywords:	Fetal medicine < OBSTETRICS, Prenatal diagnosis < OBSTETRICS, Ultrasonography < OBSTETRICS

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Manuscripts

Study protocol for a randomized controlled trial: Treatment of early intrauterine growth restriction with low molecular weight heparin (TRACIP)

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ABSTRACT

Introduction: The incidence of Intrauterine growth restriction (IUGR) is estimated at about 3% of pregnancies, and it is associated with 30% of all perinatal mortality and severe morbidity with adverse neurodevelopmental and cardiovascular health consequences in adult life. Early onset IUGR represents 20–30% of all cases and is highly associated with severe placental insufficiency. The existing evidence suggests that low molecular weight heparin (LMWH) has effects beyond its antithrombotic action, improving placental microvessel structure and function of pregnant women with vascular obstetric complications by normalizing pro-angiogenic and anti-apoptotic protein levels, cytokines, and inflammatory factors. The objective of our study is to demonstrate the effectiveness of low molecular weight heparin in prolonging gestation in pregnancies with early-onset IUGR.

Methods and analysis: This is a multicenter, triple-blind, parallel-arm randomized clinical trial. Singleton pregnancies qualifying for early (20–32 weeks at diagnosis) placental IUGR (according to Delphi criteria) will be randomized to subcutaneous (sc) treatment with bemiparin 3,500 IU/0.2 ml/day or placebo from inclusion at diagnosis to the time of delivery. Analyses will be based on originally assigned groups (intention-to-treat). The primary objective will be analyzed by comparing gestational age and prolongation of pregnancy (days) in each group with Student's t tests for independent samples and by comparing Kaplan-Maier survival curves (from inclusion to delivery, log-rank test). A linear regression model for gestational age at birth will consider the following covariates: gestational age at inclusion (continuous) and preeclampsia (binary).

Ethics and dissemination: The study will be conducted in accordance with the principles of Good Clinical Practice. This study was approved by the Clinical Research Ethics Committee (CEIC) of Sant Joan de Déu Hospital, on July 13th 2017. The trial is registered in the public registry www.clinicaltrial.gov. according to Science Law 14/2011, and the results will be published in an open access journal.

Competing interest statement: The authors report no conflict of interest.

Trial registration number: NCT03324139

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Author contributions:

EM is the project monitor

NM, AP, DO, and JS are coordinators at their respective sites.

EM is the general coordinator and PI of the project.

MC, CR, MDGR, and MDT are consultants.

FF is the co-principal investigator of the study.

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STRENGTHS AND LIMITATIONS OF THIS STUDY

- This is a multicenter, triple-blinded, randomized trial with two parallel groups.
- The primary outcome (prolongation of pregnancy) is a surrogate of perinatal health.
- The sample size limits the analysis of secondary outcomes.
- Follow-up of the offspring is limited to the neonatal period.

INTRODUCTION

BACKGROUND

Placental insufficiency and intrauterine growth restriction (IUGR)

Intrauterine growth restriction (IUGR) is defined as a failure to achieve the endorsed growth potential secondary to placental insufficiency. Its incidence is estimated at about 3% of pregnancies, and it is associated with 30% of all perinatal mortality and severe morbidity,[1] with adverse neurodevelopmental and cardiovascular health consequences in adult life.

Early onset IUGR represents 20–30% of all IUGR[1] and presents in association with early preeclampsia (PE) in up to 50% of cases[1]. This type of IUGR is highly associated with severe placental insufficiency; subsequent chronic fetal hypoxia is the major contributor to poor perinatal outcomes[2].

The key to successful placental implantation is controlled activation of the hemostatic system, which balances the process of trophoblastic invasion and protects against bleeding[3]. In PE and early onset IUGR, this vascular remodeling does not exist or is incomplete[4], which leads to placental hypoperfusion associated with endothelial cell dysfunction and subsequent coagulation, fibrin deposition, and uteroplacental thrombosis with local implications in the case of IUGR and systemic implications in the case of PE[5]. Pathological examination of the placenta shows uteroplacental thrombosis, infarctions, and fibrin deposits.

The natural history of placental insufficiency decreases fetal well-being, with a progression to hypoxia and acidosis; in clinical practice, this deterioration is monitored by Doppler study of the umbilical artery (which reflects the placental lesion) and ductus venosus (which reflects fetal cardiac diastolic failure secondary to acidosis) to individually time the delivery in an elective and adapted manner. The latency time to severe fetal deterioration is variable but is usually 3–4 weeks[6]. It is estimated that between 24 and 28 weeks, each week of prolonged gestation increases survival without sequelae by 10–15%; and between 28 and 32 weeks it increases by 5%[7]. The objective of fetal monitoring is therefore to maximize gestational age at birth while minimizing the risk of death and sequelae.

Heparin and placenta: biological plausibility

There is *in vitro* evidence that heparin improves placental perfusion and stimulates neoangiogenesis. Heparin exerts its anticoagulant function through the union and potentiation of antithrombin, and it also releases tissue inhibitory factor to the circulation[4]. It also has beneficial effects through other routes that do not have to do with its anticoagulant effect[8]: it influences trophoblast growth and development, it

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decreases apoptosis, it acts as an indirect growth factor, and it diminishes inflammation via its anti-complement effects[9,10].

Significant increases in the levels of some proteins that play important roles in placental development and angiogenesis were reported in patients treated with low molecular weight heparin, such as leptin, Ang-2, VEGFR-3, angiostatin, TIMP-1, and TNF-α[11-13]

In vitro results indicate that heparin normalizes MMP-9 levels at the level of placental microvessels[9]. MMP-9 controls trophoblastic invasion, and the decrease of its expression in trophoblasts as a response to hypoxia reduces invasiveness, which can impair placenta implantation. Low molecular weight heparin significantly increases the levels of both the pro-MMP and active forms, which increases trophoblast invasiveness and promotes angiogenesis *in vitro*.

Heparin also inhibits placental apoptosis by increasing cellular protection[14] and normalizes levels of inflammatory factors (IFN-γ, TGF-β1, IL-6, IL-8) that are elevated in the microvessels of pregnant women with vascular complications.

Much of the available evidence comes from *in vitro* models; the clinical correlations of these effects described in experimental models are poorly characterized. These data suggest that low molecular weight heparin has effects beyond its antithrombotic action, improving the microvessel structure and function of pregnant women with vascular obstetric complications by normalizing levels of proangiogenic and antiapoptotic proteins, cytokines, and inflammatory factors at the microvessel level. These changes facilitate appropriate invasion and angiogenesis activity to promote endothelial cell and trophoblast survival.

Heparin and placenta: clinical evidence

There are few high-quality clinical studies on the efficacy of heparin in gestation. A review[15] involving 10 randomized trials with 1139 patients concluded that for women at high risk of complications secondary to placental insufficiency, treatment with heparin was associated with significant reductions in perinatal mortality, prematurity before 34 and 37 weeks, and low fetal weight for gestational age. However, these studies only include pregnant women at risk, mainly with a history of placental insufficiency in a previous pregnancy. Another recent meta-analysis[16] (including six clinical trials) showed that in high-risk pregnant women treated primarily with aspirin, the addition of low molecular weight heparin reduced the incidences of PE and low birth weight for gestational age by ~50%. However, another large individual patient meta-analysis failed to find any benefit of heparin in women with high-risk pregnancy[17].

Despite the evidence of potential benefit for secondary prevention of placental insufficiency and its complications, there is scant and contradictory evidence about its benefit in pregnant women who already have placental insufficiency. It would be in this clinical indication, which is associated with greater complication risk and need for preterm birth, that the necessary number of patients to treat may prove to be more beneficial. Four clinical trials were performed in women with placental insufficiency during gestation, and they employed heterogeneous diagnostic criteria and methodological aspects and results. Kingdom *et al.*[18] and Souza *et al.*[19] randomized patients with clinical criteria for placental insufficiency who were treated with heparin or placebo and did not observe significant differences in gestational age at birth (35.6 weeks vs 34.3 weeks and 35.69 weeks vs 33.5 weeks, respectively) or perinatal morbidity and mortality. In contrast, Yu *et al.*[20,21] demonstrated significant differences in gestational age at birth (37 weeks vs 38 weeks and 36.2 weeks vs 37.1 weeks) and fetal birth weight in patients with placental insufficiency treated with heparin.

Study justification

There is good biological evidence on the potential beneficial effects of heparin on the placenta, but the clinical correlation of the effects described in experimental models is lacking. In addition, the clinical evidence of its benefit to treat gestation already complicated with placental insufficiency is weak and contradictory. Heparin has the potential to prolong gestation during a clinically important period to maximize gestational age at birth to reduce mortality and perinatal sequelae.

HYPOTHESIS
Primary hypothesis

Treatment with low molecular weight heparin (LMWH) at the time of diagnosis of early onset IUGR prolongs the latency to severe fetal impairment, increasing the gestational age at delivery of these patients.

Secondary hypothesis

i. Treatment with low molecular weight heparin at the time of diagnosis of early onset IUGR reduces neonatal morbidity.

ii. Treatment with low molecular weight heparin at the time of IUGR diagnosis improves maternal and placental biochemical, angiogenic, and inflammatory profiles.

iii. Treatment with low molecular weight heparin at the time of diagnosis of early-onset IUGR reduces placental lesions secondary to poor placental and fetal perfusion.

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OBJECTIVES

Primary objectives

To demonstrate the effectiveness of low molecular weight heparin in prolonging gestation in pregnancies with early-onset IUGR.

Secondary objectives

i. To demonstrate the effectiveness of low molecular weight heparin in reducing neonatal morbidity in pregnancies with early-onset IUGR.

ii. To demonstrate the effect of low molecular weight heparin in improving pro-angiogenic and anti-inflammatory maternal and placental profiles in pregnancies with early-onset IUGR.

iii. To demonstrate the effects of low molecular weight heparin in reducing thrombotic and ischemic placental lesions.

TRIAL DESIGN

This is a multicenter, triple-blinded, randomized, phase III trial with two groups in parallel.

METHODS

STUDY SETTING

The study will be conducted within Spanish academic hospitals with specialist experience in managing IUGR: Hospital Sant Joan de Déu (Barcelona), Hospital Clínic (Barcelona), and Hospital Clínico Lozano Blesa (Zaragoza). The first two hospitals are legally integrated in the Center of Maternal Fetal and Neonatal Medicine of Barcelona (BCNatal). Each of these hospitals has over 3000 deliveries and performs more than 10,000 fetal ultrasounds annually.

ELIGIBILITY CRITERIA

Inclusion criteria:

I. Singleton fetus.

II. Diagnosis of early onset IUGR according to the Delphi classification[22] (20–32 weeks at diagnosis) with umbilical artery Doppler with absent/reversed diastolic flow; or estimated fetal weight <10th percentile plus pulsatility index (PI) of

umbilical artery Doppler >95th percentile or estimated fetal weight <10th percentile plus mean PI of uterine artery Doppler >95th percentile.

The weeks for inclusion will be between 20-32 weeks of gestation, at the moment of the diagnosis.

Exclusion criteria:

I. Abnormal karyotype, structural abnormalities, or congenital infections

II. Treatment with LMWH or oral anticoagulants prior to inclusion

III. History of heparin-induced thrombocytopenia

IV. Active hemorrhage or increased risk of bleeding due to hemostatic changes

V. Severe hepatic or pancreatic dysfunction

VI. Organic lesions that can bleed (e.g., active peptic ulcer, hemorrhagic stroke, aneurysm, or brain tumor)

Stop treatment criteria:

I. Intolerance or hypersensitivity to bemiparin

II. Allergic reaction

III. Clinically relevant bleeding (requiring hospitalization)

IV. Requirement of LMWH per clinical indication

INTERVENTION

Patients who agree to participate and provide signed informed consent signed will be centrally randomized (via a website) through the Clinical Trials Unit of Sant Joan de Déu Hospital in Barcelona (block randomization of 10) to 3,500 IU/0.2 ml/day of subcutaneous (sc) bemiparin sc or placebo of the same presentation as the active drug, from inclusion to delivery (estimated median of 5–6 weeks, maximum of 17 weeks).

The treatment will be delivered to the patient weekly using the same presentation and labeled with the study identification and participant number.

CONTROL VARIABLES:

1. Maternal age at birth; continuous (years)

2. Smoking during gestation; continuous (cigarettes/day)

3. Maternal weight at the beginning of gestation; continuous (kg)

4. Maternal height; continuous (cm)

5. Maternal ethnic origin; categorical (Europe, Africa, South America, Maghreb, Asia, Others)

6. Parity (number of deliveries >22 weeks); discrete

7. History of PE[23]; binary (Yes/No)

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8. History of gestational hypertension; binary (Yes/No)

9. History of IUGR (birthweight below the 10th percentile)[24]; binary (Yes/No)

10. Gestational age at the inclusion in the study; continuous (weeks)

11. Diastolic blood pressure at inclusion; continuous (mmHg)

12. Systolic blood pressure at inclusion; continuous (mmHg)

13. Mean PI of the uterine arteries at inclusion and before delivery; continuous (normalized by gestational age)[25]

14. PI of the umbilical artery at inclusion and before delivery; continuous (normalized by gestational age)[26]

15. PI of the middle cerebral artery at inclusion and before delivery; continuous (normalized by gestational age)[27]

16. PI of the ductus venosus at inclusion and before delivery; continuous (normalized by gestational age)[28]

17. Hypertension in current pregnancy; binary (Yes/No).

18. PE in current pregnancy; binary (Yes/No).

OUTCOMES

Primary outcomes

Gestational age at live birth (dated by ultrasound <14 weeks by measurement of crown rump length; continuous (mm)[29]).

Prolongation of pregnancy: time from inclusion to live birth.

Secondary outcomes

1. IUGR: birthweight less than the 10th percentile for our population[24] with pulsatility index of the umbilical artery during the third trimester (on two separate occasions >48 h) higher than the 95th percentile[26]; binary (Yes/No)

2. PE[23]: diastolic blood pressure (DBP) ≥90mmHg and/or systolic (SBP) ≥140 in two separate determinations, >4 h with proteinuria >300 mg/24 h or other maternal organ dysfunction/renal insufficiency, liver involvement, neurological complications, hematological complications); binary (Yes/No)

3. Severe PE: PE with BP ≥110/160 mmHg, oliguria (<400 mL/24 h), neurological symptoms, acute pulmonary edema, persistent epigastric pain, hepatic dysfunction, analytical signs of hemolysis (lactate dehydrogenase >700 U/L), and/or thrombocytopenia (<100,000/mL); binary (Yes/No)

4. Preterm birth before 34 weeks of gestation; binary (Yes/No)

5. Emergent caesarean section for loss of fetal well-being; binary (Yes/No)

6. Birthweight; continuous (g)

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7. Neonatal acidosis (arterial pH <7.10 + base excess> 12 mEq/L); binary (Yes/No)

8. Perinatal mortality (from 22 weeks of gestation to 28 days postpartum); binary (Yes/No)

9. Days in the neonatal intensive care unit; continuous (days)

10. Neonatal morbidity (seizures, intraventricular hemorrhage grade III or IV, periventricular leukomalacia, hypoxic-ischemic encephalopathy, abnormal electroencephalogram, necrotizing entercholitis, acute renal failure (serum creatinine >1.5 mg/dL) or heart failure (requiring inotropic agents); binary (Yes/No)

11. Biomarkers in maternal blood at the time of diagnosis and delivery: sFlt and PlGF; continuous (pg/mL)

12. Biomarkers in umbilical cord blood: TNF-α, IL6, IFN-γ, FGF basic, VEGF and PlGF; continuous

13. mRNAs in trophoblast: IL6, IFN-γ, TNF-α, VEGFA, VEGFB, FGF2, and RQVEGF Receptor1; continuous

PARTICIPANT TIMELINE

Table 1 shows the participant timeline.

SAMPLE SIZE

A systematic review (PubMed and ISI Web of Knowledge) of clinical trials published in pregnant women suspected of placental insufficiency involving heparin treatment (both unfractionated and LMWH) only identified 4 studies with good or very good quality (Newcastle-Ottawa scale). The meta-analysis of these studies demonstrated very high heterogeneity under a fixed-effect model (I^2 90.4%) to estimate the difference in gestational age at birth between groups. Under a random effects model, the mean difference in gestational age at delivery was 1 week with a standard deviation of 0.9. Under these assumptions, the sample needed to detect a clinically relevant difference (fixed at 1 week) at gestational age at delivery with a power of 95% and an alpha risk of 5% was 22 patients per arm (44 patients in total). To compensate for possible withdrawals, we will require 25 per arm (50 patients).

ALLOCATION

Using an online service (http://www.randomization.com), randomization sequences will be generated in blocks of six subjects to ensure balanced distribution within study arms, stratified by participating site. Randomization sequences will be also stratified for <28 and ≥28 completed weeks and for umbilical artery Doppler with present or

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absent/reverse end diastolic flow. The allocation sequence will be internally sequestered by a Clinical Trials Unit (CTU).

At the time of the diagnosis and after enrollment, recruiting physicians will obtain the allocation group from a web-based system.

Each treatment pack will only be identified by a randomization code. The treatment allocation will only be revealed to the researchers after study completion or when clinically essential.

The Pharmacy Department of Sant Joan de Déu Hospital will provide labeling for all packs and blister sheets, ensuring complete blinding to all participants and investigators including the principal investigator, participating research doctors, project managers, and others involved in the trial.

Matching placebo will be identical to the intervention (bemiparin) in such parameters as size, physical properties, and appearance. The Pharmacy Department will keep the randomization code list confidential.

PARTICIPANT COMPLIANCE AND BLINDING PROCESS

Compliance will be assessed by trial teams by counting remaining injections at each follow-up visit and inquiring about compliance weekly.

Participants will be encouraged to report any concerns or side effects in a diary for review at each trial visit.

Blinding success will be assessed before delivery by asking the managing doctor about suspicion of treatment arm (i.e., due to the presence of bruising).

DATA COLLECTION

Patients will be recruited at the time of diagnosis of early onset IUGR, when fetal-placental Doppler study is routinely performed to obtain the mean PIs of the uterine arteries, umbilical artery, middle cerebral artery, and ductus venosus .

Maternal blood will be collected at the time of diagnosis to study angiogenic factors.

The control variables defined in the previous section will also be collected in the first visit. Participant data from this first visit and subsequent visits for this study (anonymized) will be entered into an electronic case report form (e-CRF) hosted on a secure website by each site coordinator. Logic and range rules operate in the e-CRF to minimize imputation errors.

Maternal blood, cord blood, and placenta will be collected at the time of delivery. Perinatal outcomes will be collected after delivery.

Obtaining and processing of maternal blood samples:

We will collect 5 mL maternal blood in tubes without anticoagulant. They will be transported to the laboratory within 1 hour after extraction, maintaining each center's safety guidelines for the transport of biological material. Blood tubes will be centrifuged at 1500 g for 15 minutes. Supernatant (serum) will be aspirated, stored in cryovials, properly labeled, and kept in a –80ºC freezer. Levels of PlGF and SFlt-1 (pg/mL) will be determined using an Elecsys automated platform by electrochemiluminescence (Cobas analyzers, Roche Diagnostics). The intra- and inter-assay coefficients of variation are <4% and 2.4 to 4.6%, respectively.

Collection and processing of cord blood samples:

After fetal extraction, umbilical cord venous blood will be collected in a 10-mL silicone tube without additives. Samples will be transported to the laboratory within 1 hour of extraction, maintaining our center's safety guidelines for transport of biological material. Blood tubes will be centrifuged at 1500 g for 15 minutes. Supernatant (serum) will be aspirated and stored in properly labeled cryovials in an -80ºC freezer until analysis in duplicate in the LABSCAN 100 Multiplex Analyzer (Luminex) (LUMINEX 100 IS software).

Collection and processing of placental tissue samples:

After delivery, two placental tissue biopsies will be stored in later RNA in aliquots at –80ºC until the time of analysis. DNA will be extracted from fresh placental tissue with Flexigene (Qiagen). Placentas will then be fixed in buffered formalin. After gross examination, routine samples will be obtained from each specimen: one transverse section of cord, one rolled strip of membranes, and three blocks from the placental parenchyma. Additional blocks will be taken from all macroscopic lesions. Placental findings will be reported as described in the Amsterdam Consensus[30].

Maternal blood, cord blood, and placental tissue samples will be analyzed in the *Centro de Investigación Biomédica de Aragón (CIBA), Aragon Institute for Health Research (IIS Aragón) Zaragoza, Spain.*

DATA MONITORING AND SAFETY

An independent Clinical Trial Unit will perform an offline data audit every 6 months checking for missing information and errors. The same committee will monitor safety issues. There may be unexpected adverse reactions associated with low molecular

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weight heparin. All investigators have a thorough understanding of the anticipated adverse events and the appropriate reporting process. The investigators will notify the Independent Clinical Trial Unit of adverse events, and they will report to the regulatory authority and ethics committee.

PATIENT AND PUBLIC INVOLVEMENT

Patients were not involved in any part of the design of the study.

STATISTICAL ANALYSIS

Analysis will be based on originally assigned groups (intention-to-treat). However, it is possible that there will still be some missing data at the end of the study, so sensitivity analyses will be carried out to confirm the robustness of the results.

The primary objectives will be analyzed by linear regression model for gestational age and time from randomisation to live birth, in which the following covariates are considered: gestational age at inclusion (continuous) and PE (binary). Stillbirths will be considered as Missing data will be addressed by multiple imputation methods. Stillbirth cases will be penalized in the analysis by imputing them 0 days of prolongation.

The number of patients included (~50) only allows adjustment by 2 variables to maintain power >80%.

Secondary objectives will be analyzed by:

I) Univariate analysis:

    A. Continuous variables: Student's t test (or Mann-Whitney U tests for non-normal distribution, Shapiro-Wilk's test P<0.05)

    B. Categorical variables: 2 Pearson's (or Fisher's exact test)

II) Multivariate analysis using linear regression (for continuous dependent variables) or logistic regression (for binary variables).

ETHICS AND DISSEMINATION

The study will be conducted in accordance with the principles of Good Clinical Practice. This protocol was approved by the Clinical Research Ethics Committee (CEIC) of Sant Joan de Déu Hospital on July 13th 2017. Subsequent approval by individual ethical committees and competent authority was granted. Results will be published in peer-reviewed journals and disseminated at international conferences.

Patients will be informed that her participation in the trial will be treated with the same confidentiality as their clinical documentation, but, if necessary, a member of the CEIC

of the center, an inspector appointed by the health authorities, or the clinical trial monitor may have access to it. In the data collection notebook, the patient will only be identified by her study inclusion number.

The trial has been entered in the public registry www.clinicaltrial.gov (trial registration number NCT03324139) according to Science Law 14/2011, and the results will be published in an open access journal.

DISCUSSION

The main limitation is the small sample size. However, the results will be very useful for designing larger, future trials, and it will provide mechanistic data on biomarkers in maternal blood, cord blood, and placenta.

Although prolongation of pregnancy is a useful surrogate of perinatal health, the study would be largely underpowered if we set neonatal mortality/morbidity as a primary outcome.

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Table 1: Participant timeline

		STUDY PERIOD		
	DIAGNOSIS 20–32 w	POST-ALLOCATION 20-32 w	DELIVERY	CLOSE OUT: 28 d after delivery
Eligibility screen	X			
Informed consent	X			
Allocation	X			
INTERVENTIONS				
Concealment		X		
Revealment		X		
ASSESSMENTS				
Socio-demographic data	X			
Medical history	X			
Follow-up: ultrasound biometries and Doppler assessment		X[1]		
Biomarkers in maternal blood	X		X	
Ensure compliance		X		
Biomarkers in cord blood and placenta			X	
Check side effects/ adverse events		X		
Perinatal outcome				X

[1]According to local protocol

(https://medicinafetalbarcelona.org/clinica/images/protocolos/patologia__fetal/cir-peg.pdf)

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SPIRIT ☑

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	_____1_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	_____3_____
	2b	All items from the World Health Organization Trial Registration Data Set	__------____
Protocol version	3	Date and version identifier	__--------_____
Funding	4	Sources and types of financial, material, and other support	_____3_____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1 and 3
	5b	Name and contact information for the trial sponsor	____-----_____
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	__------___
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	_____--------_____

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Introduction

| Background and rationale | 6a | Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention | _5-8_____ |

| | 6b | Explanation for choice of comparators | 5-8_____ |

| Objectives | 7 | Specific objectives or hypotheses | _____8-9_____ |

| Trial design | 8 | Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) | ___9_____ |

Methods: Participants, interventions, and outcomes

| Study setting | 9 | Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained | _____9_____ |

| Eligibility criteria | 10 | Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) | ____9-10_____ |

| Interventions | 11a | Interventions for each group with sufficient detail to allow replication, including how and when they will be administered | ___10_____ |

| | 11b | Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) | _10_____ |

| | 11c | Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) | _13_____ |

| | 11d | Relevant concomitant care and interventions that are permitted or prohibited during the trial | _10_____ |

| Outcomes | 12 | Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended | 11-12__ |

| Participant timeline | 13 | Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) | 12 and 20___ |

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| Sample size | 14 | Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations | 12_____ |
| Recruitment | 15 | Strategies for achieving adequate participant enrolment to reach target sample size | __12_____ |

Methods: Assignment of interventions (for controlled trials)

Allocation:

Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	12_____
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	__12_____
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	____12_____
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	___12 and 13____
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	___10_____

Methods: Data collection, management, and analysis

| Data collection methods | 18a | Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol | __13-14_____ |
| | 18b | Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols | ___13-14_____ |

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Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	13_____
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	__14-15_____
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	14-15__
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	14-15_____

Methods: Monitoring

Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	_____14_____
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	_____14_____
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	_____14_____
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	____14_____

Ethics and dissemination

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	___15-16_____
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	_____15-16

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Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	10_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	--------_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	15_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	3_____
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	15-16_____
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	---------_____
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	15-16_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	----------_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	---------_____

Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	---------_____
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	_------------_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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